





Article

Evaluation of Greenness of LC-MS Chromatographic Methods for Simultaneous Analysis of Mixtures of Serotonin, Dopamine, Acetylcholine, GABA and Glutamate: AGREE Tool Application

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Abstract: The analytical GREENness metric (AGREE) tool is widely used as a reliable greenness assessment method for chromatographic analyses. The AGREE tool has the ability to determine the greenness of analytical methods in terms of both quality and quantity, whereas other commonly used methods assess the greenness either quantitatively or qualitatively. Greenness profiles of six chromatographic methods for simultaneous estimations of serotonin, dopamine, acetylcholine, GABA and glutamate were assessed using AGREE and NEMI tools as a case study. The AGREE assessment tool proved to be user-friendly, and provides a full profile of assessment, hence it can be described as the tool of choice for the assessment of LC-MS chromatographic methods. For optimum application, the weights of 4 of the 12 assessment criteria were set high (weight of four) due to their importance, namely criteria number 7 (waste), number 8 (analysis throughput/number of analytes per run), number 11 (toxicity) and number 12 (operator's safety). Setting proper weights of the assessment criteria contributed significantly to the discrimination of greenness of the compared methods. The selected greenest method for the analysis of the proposed quinary mixture showed an AGREE tool pictogram with a 0.66 score. Additionally, the selected method allows simultaneous estimation of seven constituents in total. It offers high sensitivity, allowing detection of acetylcholine, serotonin and glutamate at levels as low as 2 pg, and dopamine, norepinephrine, GABA and glycine at levels as low as 10 pg, and finally offers fast analysis where all components can be analyzed within 5 min.

Keywords: AGREE tool; NEMI tool; neurotransmitters; green chemistry; serotonin; dopamine; acetylcholine; GABA; glutamate

1. Introduction

The neurotransmitters of the central nervous system (CNS) are chemical messengers that carry neurotransmission between neurons. These neurotransmitters are important for the function of the nervous system and are involved in many disorders of the CNS. The most common CNS neurotransmitters are serotonin, dopamine, acetylcholine, GABA and glutamate [1–3].

To correlate the imbalance of these neurotransmitters and the impact on brain physiology, it is important to accurately measure the concentrations of these neurotransmitters. Moreover, carrying out simultaneous analysis of these neurotransmitters can be very useful in characterizing their roles in several neurological disorders.

Various analytical techniques applied for estimating the quinary mixture of neurotransmitters were reviewed from the analytical abstracts service provided by the Royal Society of Chemistry [4]. The search identified eight chromatographic techniques for simultaneous estimation of various neurotransmitters. Quantification of six neurotransmitters using LC-MS/MS was reported by three studies. The second method was designed to detect seven neurotransmitters using MD-HILIC-MS/MS [5]. The third method was conducted to detect eight neurotransmitters using LC-MS [6,7]. There is only one report of a study quantifying ten neurotransmitters along with their metabolites [8]. All these methods involved the quantification of 5-HT, DA, GABA and Glu, and their metabolites.

In this study, the analytical GREENness metric (AGREE) tool was used to evaluate the greenness of the six selected analytical chromatographic methods for the simultaneous analysis of the five targeted neurotransmitters as a case study. The greenness assessment tool presented in this work has been found to be a more qualitatively and quantitatively efficient tool (through its numerical pictogram and coverage of all the 12 principles of green chemistry, abbreviated as SIGNIFICANCE) compared with the other greenness assessment methods such as the National Environmental Methods Index Label (NEMI), Analytical Eco-Scale Method (ESA) and Green Analytical Procedure Index (GAPI) [9]. AGREE is flexible, comprehensive and easy to use and interpret the results with good information. The calculation of the greenness involves the inputs of all principles of green analytical chemistry.

All the chromatographic methods reported allow concurrent estimation of the five selected neurotransmitters. The analytical GREENness metric (AGREE) tool was used to assess the greenness of the selected six chromatographic analytical studies. This greenness assessment tool proved to be more qualitatively and quantitatively efficient compared to the other methods of greenness assessment [9].

As a concern of the environment, green and sustainable materials have attracted significant research interest with determination to replace fossil-based materials and reduce waste production [10–13]. Green analytical methods are important as they reduce energy input, solvent use, waste production and operator's exposure [14], and avoid the use of toxic organic solvents. Currently, green chemistry principles have been included into academic programs [15,16] for training new researchers to take into account the impact of their research on the environment. The vast majority of researchers are aiming to transform their analytical techniques to comply with the principles of green chemistry. Endeavors are ongoing to apply green chemistry principles in instrumentation and analytical techniques, as it is important to develop more sustainable and efficient sample preparation techniques [17–20]. Applying green chemistry principles to sample preparation leads to substantial socioeconomic and environmental impacts [21,22].

This work has been designed as a case study to assess the greenness of the five chromatographic analytical methods used to assay the levels of the studied quinary mixture (5-HT, DA, Ach, GABA and Glu). Moreover, the study aims to identify the most sensitive, fastest and widest scope of application of the compared chromatographic methods. This will eventually provide a valuable analytical tool for neuroscience research.

2. Methods

2.1. Greenness Assessment Methods

2.1.1. The Analytical GREENness Metric Tool (AGREE)

AGREE is a newly introduced method of greenness evaluation, introduced by Pena-Pereira et al. [23]. This method addresses the 12 principles of green analytical chemistry (GAC) abbreviated as SIGNIFICANCE.

As per the calculation of greenness by AGREE tool, the following factors contribute to the greenness:

1. Pretreatment/extraction process or reagents added to the mobile phase to improve chromatographic resolution [24]. Elimination of pretreatment of samples significantly contributes to the greenness by reducing hazard to the environment and operators.

The AGREE tool takes into consideration many factors like online, offline, at line, invasiveness and remote sensing in estimating the greenness.

2. Minimum quantity and number of samples.
3. Location of the device must be as close as possible to the analysis site, which reduces time of analysis.
4. Number of steps in the analysis.
5. Use of automated and miniaturized devices.
6. Circumventing derivatization.
7. Volume of waste produced.
8. Number of analytes estimated at a time.
9. Energy consumption.
10. Source of reagents used, as renewable sources are safer.
11. Toxicity of the reagents.
12. Operator's safety.

In this method, a colorful pictogram is generated with 12 divided sections at the borders (Figure 1). Each of the 12 sections represents one of the GAC principles and is given a score that ranges from one to zero, where one means fully green (given a green color) and zero means non-green (given a red color), with grading colors in between. In the middle of the pictogram, there is an overall score which numerically represents how green the method is. The founders of the AGREE tool aimed to establish it based on solid guidelines, which include the flexibility of inputs, inclusiveness, clarity of yields and simplicity, where full explanation can be found in their work. The software that runs the analysis could be downloaded from the website provided by authors in their article [23].

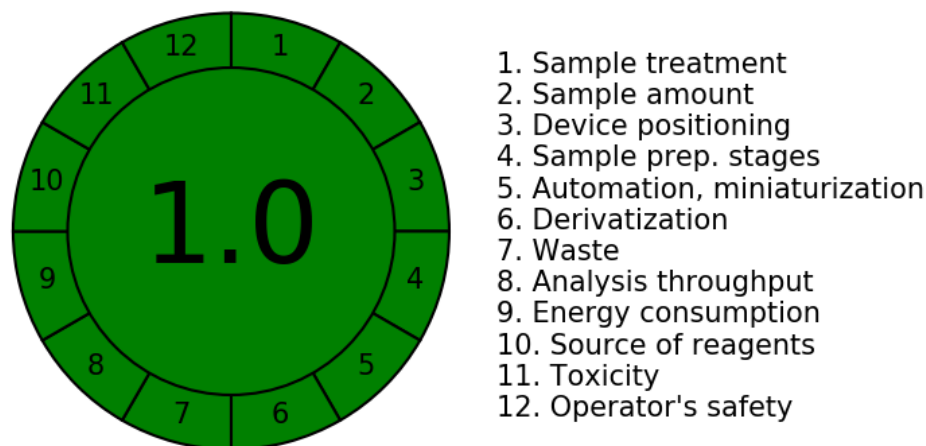


Figure 1. Greenness evaluation criteria as suggested by the AGREE method.

To allow proper comparison, the weights for the 12 evaluation criteria were reset, where the default weight given for all was two according to the instructions for use. However, we changed the weights for criteria number 7 (waste), number 8 (analysis thruput/number of analytes per run), number 11 (toxicity) and number 12 (operator's safety), where all criteria were set to have a weight of four due to their importance and ability to differentiate chromatographic methods (Figure 2).

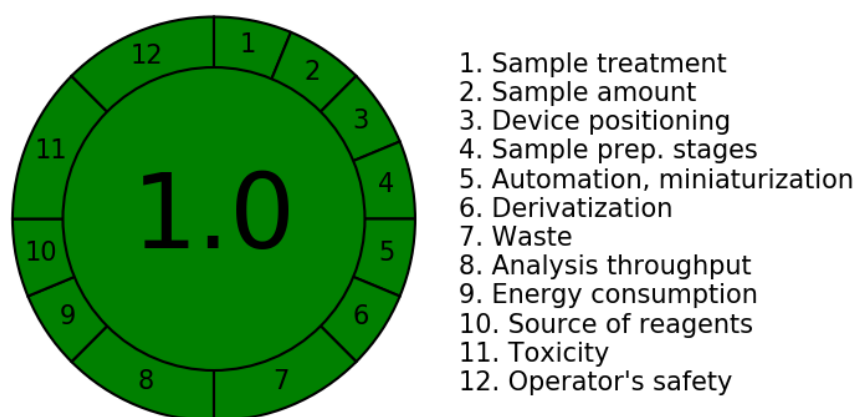


Figure 2. Greenness evaluation criteria as suggested by the AGREE method after readjusting criteria numbers 7, 8, 11 and 12 to have a weight of four.

2.1.2. National Environmental Method Index (NEMI)

NEMI, developed by the Methods and Data Comparability Board, is one of the largest databases of environmental analytical methods [25]. NEMI, which is free for scientists, comprises links to various guidelines, abstracts and whole methods [26]. The greenness profiles and approval norms are established and adjusted based on the data obtained from analytical procedures and translated to a greenness report, which includes information about the chemicals used, waste produced and pH. The generated report has four components, namely hazardous, toxic (PBT), corrosive, bio-accumulative, persistent and waste. These components are represented by quarters in a circle, where quarters are colored with different shades of green according to their greenness [27].

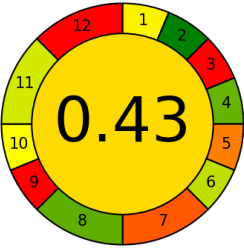
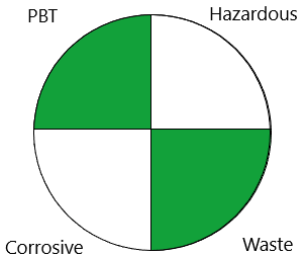
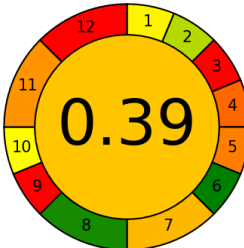
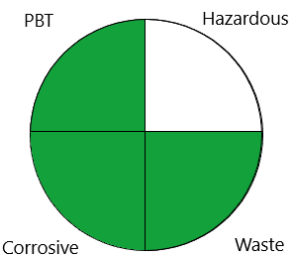

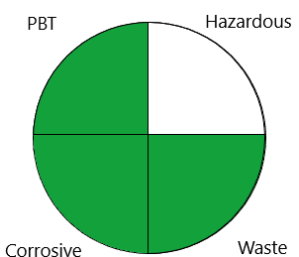
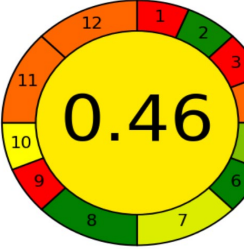
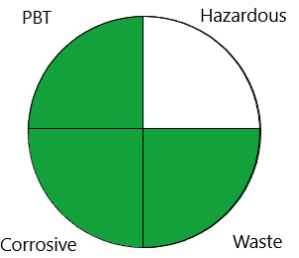
2.2. Chromatographic Methods under Investigation

A summary of the chromatographic methods used for the analysis of the five neurotransmitters under investigation is provided in Table 1 [5–7,28–30].

Table 1. Pictogram plots for the AGREE tool for the assessment of the chromatographic methods under comparison.

Method	Instrument and Chromatographic Settings	AGREE Assessment Method Pictogram Plot	NEMI Assessment Method Pictogram Plot
1	<ul style="list-style-type: none"> The analytical method reported by Ya-Bin Tang et al. [5]: Using microdialysis coupled with HICM, different neurotransmitters (Ach, 5-HT, DA, NE, Glu, GABA and glycine) were quantified. Stationary phase: Merck ZIC-HILIC column (2.1–100 mm, 3 mm; Merck Sequant, Umea, Sweden). Mobile phase: Isocratic; CH₃OH and H₂O (55: 45 v/v); 20 mM NH₄HCO₂ adjusted to pH 3.0 using CH₂O₂. Flow rate: 0.2 mL min^{−1}. Time of analysis: 5 min. 		
2	<ul style="list-style-type: none"> The analytical method reported by Qianqian Wang et al. [6]: Online microdialysis coupled with LC-MS. The analytes were Glu, aspartic acid, GABA, serine, taurine, Ach, DA and 5-HT. Stationary phase: C₁₈. Mobile phase: Water (0.12% formic acid); Methanol (0.06% formic acid) (67:33 v/v). Storage: Stock solutions stored at −80 °C. Flow rate: 0.5 mL min^{−1}. Time of analysis: 19.5 min. 		

Table 1. Cont.

Method	Instrument and Chromatographic Settings	AGREE Assessment Method Pictogram Plot	NEMI Assessment Method Pictogram Plot
3	<ul style="list-style-type: none"> The analytical method reported by Xiaozhe Zhang et al. [28]: HIC-MS was used to simultaneously measure the neurotransmitters Ach, 5-HT, DA, GABA, Glu and aspartate in the primate cerebral cortex. Stationary phase: Fused-silica capillary tubing of 200 mm i.d. Mobile phase: Gradient (0.00 to 6.00 min, 85% B to 45% B, followed by 45% B for 8 min); (A) H₂O supplemented with 20 mM NH₄HCO₂ and 1% formic acid; (B) CH₃CN. Gradient profile was used to elute the test compounds. Flow rate: 3.5 mL min^{−1}. Time of analysis: 14 min. 		
4	<ul style="list-style-type: none"> The analytical method reported by Linjia Sun et al. [7]: Eight neurotransmitters (Glu, GABA, Ach, 5-HT, DA, NE, Trp and Tyr) or their precursors in rat blood and brain were simultaneously quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Stationary phase: C18 MG column (4.6 m × 150 mm, 5 mm). Mobile phase: Gradient; (A) H₂O containing 0.1% CH₂O₂; (B) CH₃CN. Flow rate: 0.8 mL min^{−1}. Time of analysis: 15 min. 		
5	<ul style="list-style-type: none"> The analytical method reported by Cristian Gómez-Canela et al. [29]: Nine neurotransmitters (Glu, GABA, glycine, DA, NE, epinephrine, 5-HT, Ach and histamine) were simultaneously quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for multiresidue determination of neurotransmitters and their metabolites. Stationary phase: Synergy Polar-RP column. Mobile phase: 600 µL min^{−1}, binary mixtures with 0.1% formic acid in water (A) and 0.1% formic acid in MeOH (B), with systematic gradient elution. Flow rate: From 95% A and 5% B for 2 min to 30% B for 5 min. Then, the gradient was increased to 95% B in the subsequent 13 min and kept for 5 min. Initial conditions were attained in 5 min to stabilize the system. Time of analysis: 30 min. 		
6	<ul style="list-style-type: none"> The analytical method reported by Marianne Skov-Skov Bergh et al. [30]: Ten neurotransmitters or their metabolites (DA and its metabolites homovanillic acid and 3-methoxytyramine, 5-HT and its metabolite 5-hydroxyindoleacetic acid, NE, Ach, Glu and GABA) in rodent brain tissue and extracellular fluid were simultaneously measured by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Stationary phase: Acquity UPLC HSS T3; C18 column (2.1 × 100 mm, 1.8 µm particle size). Mobile phase: Gradient elution with methanol was applied and quantification was performed using multiple reaction monitoring (MRM). Flow rate: 0.5 mL/min. Time of analysis: 5.2 min. 		

3. Results and Discussion

The elucidation of the strengths and weaknesses among the twelve principles of GAC is the main advantage of the AGREE method [31,32]. Additionally, the overall score of this method is reliable and informative regarding GAC. According to literature and previous comparisons [9,33,34], and when compared to the Eco-Scale Assessment method, the AGREE method is more preferable based on the analytical method output (number of samples per hour), sample size, uses of bio-based solvents and toxic aqueous hazards. Additionally, the AGREE method is superior to the Green Analytical Procedure Index method in terms of simplicity and automation as comparable conclusions could be drawn with less effort.

Moreover, in order to optimize the performance of the AGREE tool, the most essential question remains: on what basis can the relative weight of the 12 SIGNIFICANCE sections in a pictogram be reduced or stretched? Accordingly, in this study we decided to give higher weights for criteria number 7 (waste), number 8 (analysis throughput/number of analytes per run), number 11 (toxicity) and number 12 (operator's safety), where these selected criteria were set to have a weight of four due to their importance and ability to differentiate chromatographic methods.

In this study, we assessed the greenness of 6 analytical methods using the AGREE and NEMI tools (Table 1). Method 1 was found to be the greenest with an AGREE score of 0.66, which is also consistent with the NEMI result. The greenness scores (AGREE) of the other methods were in the following order: method 5 (0.6), method 2 (0.56), method 6 (0.46), method 3 (0.43) and method 4 (0.39). The NEMI pictogram was the same for all methods except method 3, which indicates that it is not depicting any differentiation in the greenness when compared to the AGREE tool, hence using NEMI is not recommended for differentiating the greenness of chromatographic methods.

The greenness of the six chromatographic methods has been compared for all criteria as per the results of the AGREE tool and presented in Table 2. In methods 1, 2, 3 and 4, section 12 it appears red and in method 6 it appears orange, which indicates that the safety of the operator has been compromised in all methods except method 5. Section 9 (energy consumption) appears red in all methods, affecting the overall greenness. The sample amount (section 2), derivatization (section 6) and analysis throughput (section 8) appear green across all the methods, contributing to their greenness. The positioning of the device (section 3) appears red in all methods except in methods 1 and 2, affecting their greenness. The source of reagents (section 10) appears yellow in all methods, showing no discriminating effect.

In conclusion, the AGREE method was found to be a very reliable, simple, easy to use and fast greenness assessment tool. It gathers both qualitative and quantitative aspects of comparison. The 12 criteria (C) considered for the evaluation are given the same weight indicating their equal importance. Method 1 in this case study was the greenest compared to others. In method 1, which is online microdialysis coupled with hydrophilic interaction chromatography–tandem mass spectrometry (MD-HILIC–MS/MS), the sample is prepared externally with only one step (C1) and a maximum needed concentration of 400 ng/mL (C2). The measurement was online (C3) and the method has three steps (C4). In addition, this method is not an automatic or miniaturized method (C5) and there is no derivatization required (C6). The amount of waste is 1 mL which constitutes the analytes CH₃OH, H₂O and NH₄HCO₂ (C7). Seven analytes are estimated simultaneously and the analysis time is 5 min (C8). MD-HILIC–MS/MS is a high energy-consuming method (C9) in which CH₃OH used can be obtained from a bio source (C10). The amount of toxic solvent used is 0.55 mL/analysis (C11) but CH₃OH is highly flammable (C12). These criteria resulted in a final score of 0.66. A supplementary file generated by the program illustrates the calculations, Supplementary File S1.

Table 2. Comparison of greenness of methods based on AGREE tool results.

Evaluation Criteria	Method *					
	1	2	3	4	5	6
1. Sample treatment						
2. Sample amount						
3. Device positioning						
4. Sample preparation stages						
5. Automation and miniaturization						
6. Derivatization						
7. Waste						
8. Analysis throughput						
9. Energy consumption						
10. Source and reagents						
11. Toxicity						
12. Operator's safety						
Overall Greenness score out of 1	0.66	0.56	0.43	0.39	0.6	0.46

* The colors in table range from red (not green method) to green (green method).

Nevertheless, proper selection of weights given to the 12 SIGNIFICANCE criteria represents an essential issue for proper application of the AGREE tool. The four criteria that were given higher weights could have a real effect in the discrimination of the greenness of the compared methods. The greenness may be further improved following newly introduced techniques [35].

4. Conclusions

Green chemistry is a quickly developing science, and the assessment of greenness of chromatographic methods is one of the hot topics receiving attention in the analytical chemistry society. In this article, an AGREE greenness assessment tool was applied successfully for the assessment of six chromatographic methods used for simultaneous analysis of five important neurotransmitters, namely Ach, 5-HT, DA, GABA and Glu. Among the six chromatographic methods, method 1 [5] proved to be the method of choice for any analyst aiming to analyze the proposed quinary mixture for its greenness (according to the AGREE tool pictogram (0.66 score)), wide scope of application (where seven components can be analyzed), high sensitivity (where very low levels can be detected e.g., 2 pg for Ach, 5-HT and Glu, and 10 pg for DA, NE, GABA and glycine) and finally fast analysis (where all components can be analyzed within 5 min). However, the safety of the operator and energy consumption seem to be compromised in all the methods as per the AGREE greenness report. The four criteria that were given higher weights could have a real effect in the discrimination of the greenness of the compared methods. Finally, AGREE is a highly recommended tool that proved to be the tool of choice for greenness assessment where it offers both qualitative and quantitative measures of assessment. Nevertheless, attention must be paid to setting proper weights of the 12 assessment criteria and this may critically affect comparative results.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations9060147/s1>. Supplementary File S1.

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