Environment-functionality-cost balance of an analytical reagent

Hannah Hirmz[a], Frederick Verbeke[a], Bartosz Wielgomas[b], Christophe Furman[c], Emmanuelle Lipka[c], Bart De Spiegeleer[a] and Evelien Wynendaele[a*]

[a] Drug Quality and Registration (DruQuaR) group, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium.

[b] Department of Toxicology, Faculty of Pharmacy, Medical University of Gdańsk, Gdańsk, Poland.

[c] Inserm, Institut Pasteur de Lille, U1167 – Risk factors and molecular determinants of aging-related diseases, Université de Lille, Lille, France.

*Corresponding author, e-mail: Ejwynend.Wynendaele@UGent.be

Abstract

Green chemistry was originally mainly driven by organic synthetic approaches, but green analytics is slowly following, searching for ways to reduce volumes, cost and ecotoxicity of analytically used chemicals and lowering energy usage. The emphasis is currently focussed on techniques such as miniaturisation, on-line spectroscopy, or the chemicals directly used in the analytical process, *e.g.* chromatographic solvents. However, almost no attention has yet been paid to the analytical reagents, and more specifically, the way they are produced and used in greening analytics. In the analysis of low level analytes, such as peptides in the biomedical area, a prominent challenge is their possible adsorption to glass or plastic consumables used during analysis. In this research, a recently developed anti-adsorption diluent based on bovine serum albumin, acetonitrile and formic acid, was investigated towards greener alternatives. The 12 principles of green chemistry were applied, but also the anti-adsorption functionality and cost-efficiency were taken into account to obtain a more holistic sustainability view. A Derringer desirability function was used to convert these 3 aspects into one overall 'fit-for-purpose' score, from which it was concluded that replacing acetonitrile by (denatured) ethanol is the most optimal choice, whilst maintaining bovine serum albumin as protein source.

Keywords

Green analytical chemistry, sustainability, anti-adsorption solution, peptidomics

1. Introduction

Adsorption of analytes can render analytical method development extremely difficult, causing false negative results and requiring additional testing, thereby increasing the cost and environmental burden. Peptides are a crucial class of biomarkers, which are mostly present in biofluids at low concentrations. Their spatio-temporal levels, linked to their functionality, are studied in the so-called "peptidomics". However, one of the main challenges is the adsorption to various consumables used in their analysis. As a result, unsatisfactory peptide recovery and unnecessary elevated limits of detection and quantification, and in worst case, unreliable results and false conclusions, are obtained (Dunning, 2019). Various generic approaches to alleviate peptide adsorption have previously been reported, for example the use of a blocking agent (Jung, 2019) or via silanizing the analytical consumables (Goebel-Stengel et al., 2011). Structural analogues of the peptides of interest have also proven to be effective in reducing peptide adsorption (Fouda et al., 1991). However, many of these generic approaches render the analytical solution mass spectrometry-incompatible. A liquid chromatography-mass spectrometry compatible anti-adsorption diluent was recently reported, and the functionality of this anti-adsorption diluent was demonstrated with various peptides (Donohue et al., 2021; Verbeke et al., 2020).

The use of excessive amounts of hazardous solvents and reactants, the generation of waste and use of large amount of energy: these are only a few undesirable characteristics of synthetic bulk chemistry. In order to make a change, the concept of 'green chemistry' arose about two decades ago with Anastas and Warner's 12 principles of green chemistry as the foundation (Anastas and Warner, 1998). These principles include but are not limited to the reduction or replacement of hazardous catalysts, minimization of waste generation, reduction or replacement of hazardous solvents and the defossilization of chemicals and decarbonization of energy (Anastas and Warner, 1998; Sheldon and Brady, 2022). These principles have not only practically been applied in the industry to ecologically optimize production processes, but also some general frameworks have expanded from them. For example, the American Chemical Society developed the Solvent Selection Tool (American Chemical Society, 2018). This tool, based on principal component analysis, allows selection of green alternative

61 solvents with chemical properties similar to the ones of the solvent originally used. Moreover, several
62 other scoring guides have been developed by *e.g.* Astra Zeneca, GlaxoSmithKline (Prat et al., 2014) and
63 more recently the CHEM21 solvent guide (Prat et al., 2015), which score the most commonly used
64 solvents on environmental, safety and health parameters after which they are ranked accordingly.
65
66 The trend to "greenify" chemistry has found its way into other chemical branches, such as analytical
67 chemistry. Because Anastas' 12 principles are mainly focused on synthetic bulk chemistry and therefore
68 less applicable to the small-scale analytical chemistry, the latter discipline has been provided with its
69 own 12 principles of green analytical chemistry (Gałuszka et al., 2013). Therefore, green analytical
70 chemistry could be seen as a different branch of green chemistry, rather than its extension. When
71 greenifying an analytical method, focus is mostly put on the reduction and elimination of toxic solvents,
72 the reduction of waste production, the reduction of sample treatment steps and miniaturization of the
73 methods (Claux et al., 2021; Dhoru et al., 2020; Dogan and Tobiszewski, 2020; Gionfriddo, 2020;
74 Michalski and Pecyna-Utylska, 2020). Similar to green chemistry, theoretical frameworks in the form
75 of metric systems have been developed. This allows for quantification and direct comparison of the
76 analytical methods. The most used green analytical chemistry metrics are the Analytical Eco-Scale
77 (Gałuszka et al., 2012), the Green Analytical Procedure Index or GAPI (Płotka-Wasylka, 2018) and the
78 Analytical Greenness Calculator Metric or AGREE (Pena-Pereira et al., 2020), each with their own
79 advantages and disadvantages (Sajid and Płotka-Wasylka, 2022).
80
81 Recently, the position of green analytical chemistry as a tunnelled discipline has been questioned. It has
82 been argued that green analytical chemistry should be a share in the bigger picture of sustainable
83 development. Sustainability does not only take into account the environment, but also societal and
84 economic aspects (Płotka-Wasylka et al., 2021). Considering green analytical chemistry, when
85 optimizing a method, the main focus has been put on ecological optimization while functional and
86 practical optimization were often neglected. Hence, it seems that greenifying a method is the ultimate
87 goal instead of being one of many factors important in method optimization. This is why Nowak et al.
88 (2021). proposed the concept of 'white analytical chemistry' as an extension of green analytical

chemistry. White analytical chemistry aims to reconcile the principles of green analytical chemistry ('green') with the analytical functionality ('red') and the practical feasibility ('blue') of the method. The ideal method is a balanced combination of the 3 colours green, red and blue, resulting in white in reference to the RGB model.

The development, production and application of analytical reagents such as this anti-adsorption diluent falls between production and analytics: its production could be seen as a part of green chemistry, while its use is part of green analytical chemistry. Although it could be considered part of the analytical method life cycle, the green evaluation and optimization of analytical reagents is often overlooked in green optimizing analytical procedures. Additionally, functionality and feasibility of the product or analytical procedure are often also overlooked. Therefore, in this research, the original anti-adsorption diluent as well as alternative compositions were scored on their greenness, functionality and cost-efficiency which was ultimately combined in a single 'fit-for-purpose' score using a Derringer desirability function.

2. Material and Methods

2.1. Materials and reagents

The peptide EMRKSNNNFFHFLRRI was purchased from GL Biochem (Shanghai, China). Acetonitrile (ACN) (LC-MS grade), formic acid (FA), ammonium formate (NH_4HCO_2), trifluoroacetic acid (TFA) (both LC-MS grade), methanol (LC-MS grade), isopropanol (IPA) (LC-MS grade), water (LC-MS grade) and dimethylsulfoxide (DMSO) (gas chromatography headspace grade) were all obtained from Biosolve (Valkenswaard, The Netherlands). Denatured ethanol (denatured with 1% V/V isopropanol and 1% V/V methylethylketone (MEK) + 1,5-Diazabicyclo[4.3.0]non-5-ene (DBN)), i.e. Disolol®, was obtained from Chemlab Analytical (Zedelgem, Belgium). Protein LoBind centrifugation tubes were purchased from Eppendorf (Hamburg, Germany). (Ultra) high performance liquid chromatography ((U)HPLC) glass vials with pre-slit silicon septum and (U)HPLC vial inserts were purchased from Waters (Milford, MA, USA). Bovine serum albumin (BSA), ovalbumin (OVAL), and lactalbumin (LAC) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Anti-adsorption diluent preparation

The anti-adsorption diluent was prepared as previously described (Verbeke et al., 2020). In brief, approximately 0.5 g BSA was dissolved in 50 mL H_2O + 0.1% FA. 25 mL of this solution was transferred into a 100 mL volumetric flask and diluted to 100 mL with ACN + 0.1% FA. This solution was heated to 95°C for 5 minutes and cooled down for 30 minutes on ice. After centrifugation for 15 minutes at 20000g at 5°C. 33.3 mL supernatant was then diluted to 50.0 mL with H_2O + 0.1% FA. The obtained DruQuaR diluent was stored for a maximum of 14 days at 6°C.

Adaptations are made in (I) protein source, (II) kind of acid or its presence, (III) type of organic solvent or composition, and/or (IV) heating. The investigated anti-adsorption diluents and the presence/absence of the aforementioned variables are mentioned in **Table 1**.

Table 1: Overview of the investigated anti-adsorption alternatives. An X indicates the constitution of the considered anti-adsorption diluent alternative.

Condition	Protein[1]	Acid[2]		Weak base[3]	Organic solvent[4]			Heating
		FA	TFA	NH_4HCO_2	Acetonitrile	IPA	Ethanol	
1	BSA	X			X			X
2		X				X		X
3		X					X	X
4					X			X
5			X		X			X
6				X	X			X
7		X			X			
8	OVAL	X			X			X
9		X				X		X
10		X					X	X
11			X		X			X
12	LAC	X			X			X
13		X				X		X
14		X					X	X
15			X		X			X
16[5]	BSA	X			X			X
17[6]		X			X			X

[1]: With BSA = Bovine Serum Albumin, OVAL = Ovalbumin, and LAC = Lactalbumin.
[2]: FA = formic acid (0.1% m/V ≈ 21.73 mM; pH (calculated value in water) = 2.7); TFA = trifluoroacetic acid (0.1% m/V ≈ 8.77 mM; pH (calculated value in water) = 2.1).
[3]: NH_4HCO_2 (10 mM ≈ 0.06 % m/V; pH (calculated value in water) = 7).
[4]: Supplemented accordingly with acid/base. IPA stands for isopropanol and ethanol for denatured ethanol.
[5]: Condition 16 is similar to condition 1 but uses less ACN (50% V/V ACN/water + 0.1% m/V FA) for protein precipitation.
[6]: Condition 17 is similar to condition 1 but uses more ACN (90% V/V ACN/water + 0.1% m/V FA) for protein precipitation.

Regarding ovalbumin and lactalbumin, also 0.5 g of protein was weighed in a 50 mL volumetric flask.

Regarding the conditions containing no acid/base, water and ACN as such were used. Water and ACN in the ammonium formate condition was supplemented with ammonium formate to a concentration of 10 mM. TFA was supplemented to a concentration of 0.1% m/V in water and ACN (*i.e.* 8.77 mM).

AAD was also prepared using less ACN (50% V/V ACN/water + 0.1% m/V FA) and more ACN (90% V/V ACN/water + 0.1% m/V FA) for protein precipitation in the second step of AAD production. The BSA concentration was identical in the second step (*i.e.* 0.25 g BSA/100 mL) as compared to 'classical' AAD (which uses 75% V/V ACN):

1. 50%-condition (*i.e.* condition 16): 25.0 mL of a 0.5 g BSA/50 mL in water + 0.1% m/V FA (*i.e.* 21.7 mM) was transferred to a 100.0 mL volumetric flask. Additionally, 25.0 mL water + 0.1% m/V FA was added and ACN + 0.1% m/V FA was added to 100.0 mL (*i.e.* 0.25 g BSA/100 mL).
2. 90%-condition (*i.e.* condition 17): 10.0 mL of a 1.25 g BSA/50 mL in water + 0.1% m/V FA was transferred to a 100.0 mL volumetric flask and ACN + 0.1% m/V FA was added to 100.0 mL (*i.e.* 0.25 g BSA/100 mL).

Six technical replicates per AAD condition were analysed with the aforementioned peptide and the conditions were divided over 2 analytical runs which took approximately 24 h per run. The peptide stock solution was the same for both runs.

2.3. Chromatography and mass spectrometry

An Acquity H-class quaternary solvent manager, connected to an Acquity Xevo TQ-S triple quadrupole mass spectrometer (all Waters, Milford, MA, USA). The UHPLC-MS/MS method used was reported before (Verbeke et al., 2020).

3. Results and discussion

3.1. Greenness evaluation

160 The 12 principles of green chemistry by Anastas and Warner (1998) were the most important inspiration
161 for the assessment of the AAD alternatives. Five of the twelve principles were translated to fit the
162 specific aims, *i.e.* principle 1: prevention of waste, principle 3: less hazardous chemical syntheses,
163 principle 5: safer solvents & auxiliaries, principle 6: design for energy efficiency and principle 12:
164 inherently safer chemistry for accident prevention. The reason for exclusion of the other principles was
165 that these were not considered relevant for this application, namely the assessment of a small laboratory
166 scale production of an analytical adjuvant.

167 3.1.1. Principle 1: waste prevention

168 Waste prevention can be quantified by considering the ratio of mass used during the production of the
169 diluent to the mass of the final AAD, also called the Process Mass Intensity (PMI), which is defined by
170 the following equation (Jiminez-Gonzalez et al., 2011):

171

172 $$PMI = \frac{Total\ mass\ used\ in\ process\ (g)}{Mass\ of\ final\ product\ (g)}$$

173

174 The PMI was calculated for the different AAD alternatives taking into account the abovementioned
175 amounts of organic solvent, water and protein used during preparation. The small amount of acid or base
176 used was considered negligible. The non-reusable LoBind tubes were also not taken into account. This
177 approximation is justified because in the case of the AADs, the PMI is not a discriminating greenness
178 factor: for all ADD alternatives, a PMI of 1 is found as all solvents and reagents are used for the
179 production of the ADD, with only a negligible left-over sediment after centrifugation.

180 3.1.2. Principle 3: less hazardous chemical syntheses

181 Whenever feasible, chemical syntheses or productions should be designed to use substances that possess
182 little or no human and environmental toxicity. To assess this, a quantification method was developed
183 based on the Globally Harmonised System of Classification and Labelling of Chemicals (GHS), a
184 labelling and categorization requirement for every chemical in the European Union (European Union,
185 2008). Briefly, each AAD composition is awarded a 'Hazard score' which is a summation of individual

product scores. The product scores on their part are a summation of individual H-phrase scores. The H-phrase scores are based on the GHS hazard statements or "H-phrases" that can be found in the Safety Data Sheets of chemical products. These hazard statements are linked to a hazard class and category, to which a pictogram and signal word are assigned, indicating the severity of the hazard. These pictograms and signal words are converted into respectively the pictogram score and signal word score, the allocation of which can be found in **Table 2**. The multiplication of the pictogram and severity word scores results in the H-phrase score which is calculated per H-phrase concerning health or environment as found in the Safety Data Sheet. The pictogram scores are inspired by the Green Star (GS) concept as described by Ribeiro et al (2010). who attribute scores to the different hazard symbols to determine the safety of product. However, the pictograms on their own do not reflect the severity of the hazard. It was therefore decided to multiply the pictogram score with the severity word score, the latter being inspired by the Analytical Eco-Scale which multiplies the number of pictograms by the severity word (Gałuszka et al., 2012). The idea to calculate this score per H-phrase and not per pictogram emerged from the fact that one pictogram can represent either one or multiple H-phrases. Using only the pictograms for evaluation could thus not discriminate whether one or multiple hazards from the same category are attributed to the product. If a H-phrase has no signal word or pictogram, it gets assigned a score of 1. The H-phrase scores are then summed per product, *i.e.* solvent, protein or acid, resulting in a product score. If the Safety Data Sheet does not indicate any health or environmental hazard, a product score of 0 is allocated to the product. The product scores are corrected for their respective mass fractions in the AAD production. Per AAD, the Hazard score is then calculated by summing the individual product scores of the products used in the respective AAD. The calculations concerning principle 3 can be found in **Table 3**.

209 **Table 2: Scoring system regarding principle 3**

Pictogram score			
Pictogram	**Score**	**Pictogram**	**Score**
 **Exclamation Mark** May cause immediate health effect - eye, skin, respiratory	2	 **Environment** Hazardous to the environment	3
 **Skull and Crossbones** Acute toxicity via oral, dermal, or inhalation	3	 **Health Hazard** Aspiratory or respiratory hazard, carcinogenecity, mutagenicity	2
 **Corrosion** Corrosive, skin damage, eye damage	3		

Severity word score	
Severity word	**Score**
warning	1
danger	2
H-phrase score = Pictogram score x Severity word score	
H-phrase without pictogram/severity word	1

210

211

212

Table 3: Hazard score calculations per AAD condition. The hazard score is the sum of the individual input product scores.

Condition	Solvent score	Solvent corrected	Protein score	Protein corrected	Acid/base score	Acid/base corrected	Hazard score
1	8	3.52	0	0.00	14	0.01	**3.53**
2	4	1.76	0	0.00	14	0.01	**1.77**
3	0	0.00	0	0.00	14	0.01	**0.01**
4	8	3.52	0	0.00	-	-	**3.52**
5	8	3.52	0	0.00	15	0.02	**3.54**
6	8	3.52	0	0.00	2	0.00	**3.52**
7	8	3.52	0	0.00	14	0.01	**3.53**
8	8	3.52	4	0.01	14	0.01	**3.54**
9	4	1.76	4	0.01	14	0.01	**1.78**
10	0	0.00	4	0.01	14	0.01	**0.02**
11	8	3.52	4	0.01	15	0.02	**3.54**
12	8	3.52	0	0.00	14	0.01	**3.53**
13	4	1.76	0	0.00	14	0.01	**1.77**
14	0	0.00	0	0.00	14	0.01	**0.01**
15	8	3.52	0	0.00	15	0.02	**3.54**
16	8	2.26	0	0.00	14	0.01	**2.27**
17	8	4.33	0	0.00	14	0.01	**4.34**

3.1.3. Principle 5: safer solvents and auxiliaries

Solvents share the largest portion of the mass used in chemical synthesis and production, with fractions going up to 80%. It is therefore logical that reducing or eliminating hazardous solvents is an important factor in green chemistry. Over the course of the years, several solvent guides have been developed by *e.g.* Astra Zeneca, GlaxoSmithKline, or the Green Chemistry Institute Pharmaceutical Roundtable (GCI-PR) to help with the selection of greener solvent alternatives (Prat et al., 2014). More recently, Prat et al. (2015) provided the CHEM21 selection guide. This solvent guide can be considered a compilation and combination of the beforementioned solvent guides, and is the current recommendation of the GCI-PR. It was therefore decided to use this solvent guide for the evaluation of the AADs.

The CHEM21 solvent guide scores 51 of the most used solvents on safety, health and environmental criteria. The scores for each criterium range from 1 to 10, the higher scores being attributed to the more hazardous solvents. In this evaluation, a solvent score is attributed to each AAD alternative, according

to their used solvent. This score is the sum of the individual safety, health and environmental scores, and is corrected for the fraction of solvent used in the precipitation step. The higher the solvent score, the more hazardous the solvent. The safety score is aligned with the GHS classification system, more specifically the flammability hazard based on the flash point and boiling point. However, the CHEM21 guide makes a finer distinction by involving the auto-ignition temperature, the accumulation of electrostatic charges and formation of explosive peroxides. The health score reflects the occupational hazard, taking into account *i.a.* the boiling point of the solvents: the lower the boiling point, the more unsafe the solvent. The environmental score is based on acute environmental toxicity and the bio-accumulation potential, highlighted by their respective statements in the GHS system. The solvents are then classified as recommended, problematic or hazardous. Some solvents such as acetonitrile were reassessed in the ranking following internal in-depth expert discussions because their hazards were believed to be underestimated by CHEM21 evaluation only (Prat et al., 2015). Because of this reason, a correction for ACN was implemented by adding 2 points. The evaluation of the AAD conditions can be found in **Table 4**. Water was not included in this evaluation of organic solvents as the scope of these experiments was to search more environmentally friendly, more functional and/or more affordable alternatives to ACN, BSA and FA. This analysis could thus be regarded as a qualitative analysis with categorical factors rather than a qualitative one with continuous factors..

Table 4: **Solvent evaluation for each AAD condition.**

Condition	Safety	Health	Env	ACN correction	Sum	Fraction	Solvent score
1	4	3	3	2	12	0.75	**9.00**
2	4	3	3	-	10	0.75	**8.00**
3	4	3	3	-	10	0.75	**8.00**
4	4	3	3	2	12	0.75	**9.00**
5	4	3	3	2	12	0.75	**9.00**
6	4	3	3	2	12	0.75	**9.00**
7	4	3	3	2	12	0.75	**9.00**
8	4	3	3	2	12	0.75	**9.00**
9	4	3	3	-	10	0.75	**8.00**
10	4	3	3	-	10	0.75	**8.00**
11	4	3	3	2	12	0.75	**9.00**
12	4	3	3	2	12	0.75	**9.00**
13	4	3	3	-	10	0.75	**8.00**
14	4	3	3	-	10	0.75	**8.00**

15	4	3	3	2	12	0.75	**9.00**
16	4	3	3	2	12	0.50	**6.00**
17	4	3	3	2	12	0.90	**11.00**

It may be argued that principle 5 shows overlap with principles 3 and 12 (*cfr.* infra) and that the solvents are evaluated twice in similar ways. This is a rightful concern; however, it is not unjustified to do so: extra evaluating the solvents increases their portion in the overall greenness evaluation. The evaluation methods also show overlap as CHEM21 used in principle 5 is inspired by the GHS classification system used in principle 3. Nevertheless, some differences are observed in terms of the depth of the use of the GHS classification system and the extra added terms such as boiling point or auto-ignition point in CHEM21. The fact that the evaluations do differ, can also be deducted from the score tables. Additionally, by evaluating the solvents in multiple and different ways, more balanced expert opinion is added to the evaluation.

3.1.4. Principle 6: design for energy efficiency

Energy efficiency is often an overlooked subject in chemistry, but the consumption of energy should be recognised for its impact, both economic and environmental, and should be minimized as much as possible. For the evaluation of the AAD, the two most straightforward and relevant energy demanding steps were taken into account: heating and centrifugation. Both devices have an equal power of 300W, but the centrifugation step takes approximately 3 times longer than the heating step. The energy consumed in both steps as well as the accordingly attributed energy scores for the different AAD compositions can be found in **Table 5**. The energy score is the total energy consumed divided by 100.

Table 5: Energy score for the different AAD conditions. The energy score is the sum of the heating and centrifugation scores.

Condition	Heating (Wh)	Centrifugation (Wh)	Energy score
1	25	75	**1.00**
2	25	75	**1.00**
3	25	75	**1.00**
4	25	75	**1.00**
5	25	75	**1.00**
6	25	75	**1.00**
7	0	75	**0.75**
8	25	75	**1.00**
9	25	75	**1.00**
10	25	75	**1.00**
11	25	75	**1.00**
12	25	75	**1.00**
13	25	75	**1.00**
14	25	75	**1.00**
15	25	75	**1.00**
16	25	75	**1.00**
17	25	75	**1.00**

3.1.5. Principle 12: inherently safer chemistry for accident prevention

Substances and their form used in a chemical process should be selected to minimize the potential for chemical accidents, including fire and explosions. To evaluate the AADs on this principle, a quantification system comparable to the one of principle 3 was developed, *i.e.* based on the scoring of the GHS concept in combination with the Analytical Eco-Scale. However, in this case, the GHS pictograms concerning flammability and reactivity are taken into account. The scoring system concerning principle 12 can be found in Supplementary Table 1 and the scoring of the AADs can be found in **Table 7**.

Table 7: Safety score for the different AAD conditions

Condition	Solvent	Solvent corrected	Protein	acid/base	acid/base corrected	Safety score
1	6	2.64	0	3	0.00	**2.64**
2	6	2.64	0	3	0.00	**2.64**
3	6	2.65	0	3	0.00	**2.65**
4	6	2.64	0	-	-	**2.64**
5	6	2.64	0	0	0.00	**2.64**
6	6	2.64	0	0	0.00	**2.64**
7	6	2.64	0	3	0.00	**2.64**
8	6	2.64	0	3	0.00	**2.64**
9	6	2.64	0	3	0.00	**2.64**
10	6	2.65	0	3	0.00	**2.65**
11	6	2.64	0	0	0.00	**2.64**
12	6	2.64	0	3	0.00	**2.64**
13	6	2.64	0	3	0.00	**2.64**
14	6	2.65	0	3	0.00	**2.65**
15	6	2.64	0	0	0.00	**2.64**
16	6	1.69	0	3	0.00	**1.69**
17	6	3.24	0	3	0.00	**3.24**

3.1.6. Excluded principles

Only 5 of the 12 principles of green chemistry were included in our evaluation as the other 7 were considered not appropriate for this application. The 2nd principle of green chemistry, 'atom economy', states that the atoms or mass of the reactants in a chemical synthesis reaction should be maximally incorporated in the final product. In case of the AAD, the output product is a mixture of the input materials. No new chemical is formed as no chemical reaction *sensu stricto* takes place. Therefore, an atom balance is irrelevant. It is for the same reasoning that principles 4, 8, 9 and 10 are not applicable. Principle 4 'design benign chemicals' and principle 10 'design for degradation' focus on respectively the designed safety and degradability of the products resulting from the chemical reaction. Principle 8 and 9 deal with derivatization and catalysis which are inherently linked to chemical synthesis. None of the latter 4 principles is relevant if no chemical reaction takes place. Principle 7 on the use of renewable feedstocks and principle 11 on the real-time process analysis are not relevant in a small-scale analytical laboratory (Anastas and Warner, 1998).

3.1.7. Greenness analysis

To come to an overall greenness evaluation, the geometric mean from the separate scores is calculated as this is an appropriate central tendency measure when different unit scales are used. (**Table 8**). Equal weights were attributed to the different parameters as they were all considered equally important. The lower this overall greenness score, the better green profile.

Table 8: Complete greenness analysis for the different AAD conditions. The total greenness score is the geometric mean of the separate principle scores.

Condition	Principle 1	Principle 3	Principle 5	Principle 6	Principle 12	Greenness
1	1	3.53	9.00	1.00	2.64	2.43
2	1	1.77	8.00	1.00	2.64	2.04
3	1	0.01	8.00	1.00	2.65	0.77
4	1	3.52	9.00	1.00	2.64	2.42
5	1	3.54	9.00	1.00	2.64	2.43
6	1	3.52	9.00	1.00	2.64	2.42
7	1	3.53	9.00	0.75	2.64	2.29
8	1	3.54	9.00	1.00	2.64	2.43
9	1	1.78	8.00	1.00	2.64	2.04
10	1	0.02	8.00	1.00	2.65	0.84
11	1	3.54	9.00	1.00	2.64	2.43
12	1	3.53	9.00	1.00	2.64	2.43
13	1	1.77	8.00	1.00	2.64	2.04
14	1	0.01	8.00	1.00	2.65	0.77
15	1	3.54	9.00	1.00	2.64	2.43
16	1	2.27	6.00	1.00	1.69	1.87
17	1	4.34	11.00	1.00	3.24	2.73

Some scores (*i.e.* solvent, energy and safety) do show little variability in this specific application. The little variability in solvent scores between EtOH and IPA is not surprising, as they are two very similar alcohols. Also, the little variability in the energy scores is not surprising as the different AADs are prepared in the same way, using the same equipment. Finally, because of the similar hazards in the type of solvents and proteins respectively, only the acids and fraction of organic solvent used contribute to the little variability in the safety score. Nevertheless, because of assuring a complete view on this approach, we have kept these within our analysis as in other applications, these scores might significantly differ. It is clear that in general, when a score shows little variability in its values within the experimental region, then it has only a minor influence on the end-result.

Some conditions are significantly greener than the original condition (**Table 9**), with conditions 3 (bovine serum albumin, ethanol and formic acid) and 14 (lactalbumin, ethanol and formic acid) as equal frontrunners. Thus, changing the solvent from acetonitrile to ethanol is beneficial for the greenness of the AAD. Although less significant, changing the solvent from acetonitrile to isopropanol, decreasing the concentration of acetonitrile or dropping the heating step also decreases the greenness score and therefore improves the greenness.

Some conditions show to be less green alternatives than the original AAD with as most extreme condition, condition 16 with 90% ACN instead of 75% ACN, indicating that the amount of solvent used plays a significant role in the greenness of the AAD. Regarding the AAD conditions using denatured ethanol, changing the protein to ovalbumin renders the AAD less green, due to an increased score for principle 3. This increase is a result of the inhalation hazard mentioned on the Safety Data Sheet of the used ovalbumin, while the other proteins have no mentioned health hazards. A change of acid has no influence on the greenness of the AAD.

3.2. Greenness-functionality-cost balance

Without being functional or affordable, a greener AAD would be quite useless. It is therefore important that, next to greenness, the functionality and affordability of the AAD are also optimized to obtain the best balance of those 3 responses. The simultaneous consideration of those 3 responses is required to determine the optimal AAD. This multi-response problem can be approached by the desirability function as defined by Derringer (Derringer and Suich, 1980). The response variable Y_i is transformed into a dimensionless desirability scale d_i following the linear desirability function:

$$d_i = \begin{cases} 0 & Y_i \leq Y_{min} \\ \dfrac{Y_i - Y_{min}}{Y_{max} - Y_{min}} & Y_{min} < Y_i < Y_{max} \\ 1 & Y_i \geq Y_{max} \end{cases}$$

As the desirability of the response increases, the corresponding d_i value increases with $0 \leq d_i \leq 1$. The individual desirabilities are then combined in the geometric mean, with each desirability having an equal weight (Derringer and Suich, 1980; Van Dorpe et al., 2011):

$$D = (d_1 \times d_2 \times \ldots \times d_k)^{1/k}$$

This single value of D, which falls in the [0,1] interval, gives the overall desirability assessment of the combined response levels. D will increase as the balance of the responses becomes more favourable while if any $d_i = 0$ then D = 0 (Derringer and Suich, 1980; Van Dorpe et al., 2011).

In case of the AAD evaluation, three responses were transformed and optimized: the greenness (d_1), the functionality (d_2) and the cost (d_3). While the functionality was maximized, the greenness score and cost were minimized. Minimization of a response value Y_i is equivalent to maximization of its opposite $-Y_i$. For every response, the Y_{min} and Y_{max} values were chosen so that the minimal and maximal experimental values were respectively the 10% and 90% value of the linear range of the function. The desirability calculations can be found in **Table 9**.

Table 9: Derringer desirability calculations per AAD condition. Functionality is represented as a percentage compared to the original AAD (condition 1, set at 100%). The cost is calculated per batch of 150 mL. The total desirability D is calculated by taking the geometric mean of d_1, d_2 and d_3.

Condition	Greenness	d_1	Functionality	d_2	Cost (€)	d_3	D
1	2.43	0.23	100	0.39	5.94	0.19	**0.26**
2	2.04	0.38	90.4	0.35	4.83	0.39	**0.37**
3	0.77	0.90	107.6	0.42	2.76	0.76	**0.66**
4	2.42	0.23	83.0	0.32	5.70	0.23	**0.26**
5	2.43	0.23	228.3	0.89	6.00	0.18	**0.33**
6	2.42	0.23	0.72	0.00	5.76	0.22	**0.05**
7	2.29	0.28	46.9	0.18	5.94	0.19	**0.21**
8	2.43	0.23	75.0	0.29	5.21	0.32	**0.28**
9	2.04	0.38	131.2	0.51	4.10	0.52	**0.47**
10	0.84	0.87	111.2	0.43	2.03	0.89	**0.70**
11	2.43	0.23	0.0	0.00	5.26	0.31	**0.00**
12	2.43	0.23	53.46	0.21	5.15	0.33	**0.25**
13	2.04	0.38	89.0	0.35	4.04	0.53	**0.41**
14	0.77	0.90	110.2	0.43	1.97	0.90	**0.70**
15	2.43	0.23	149.6	0.58	5.20	0.32	**0.35**
16	1.87	0.45	78.0	0.30	5.09	0.34	**0.36**
17	2.73	0.10	51.9	0.20	6.45	0.10	**0.13**

. The functionality is of critical importance and thus taken into account in the Derringer analysis. Functionality d_2 is defined by the mean peak area of the six technical replicates per AAD composition and is then compared relatively to the mean peak area of the original AAD, which is set at 100%. For

362 the AAD with ovalbumin and TFA (condition 11), the ovalbumin did not dissolve nor became a
363 suspension, but started clumping instead. Therefore, this condition was not further processed nor
364 analyzed using LC-MS/MS for its functionality, so its functionality is set at 0.00.
365
366 Acetonitrile in combination with formic acid and the alternative proteins, especially with ovalbumin,
367 seem to be less optimal choices than the original AAD with regards to functionality. However, this
368 changes when combining acetonitrile with bovine serum albumin and trifluoroacetic acid, giving the
369 most optimal functionality of all conditions showing a peak area of 228% compared to the original AAD.
370 The second best option with regards to functionality would be the AAD containing isopropanol,
371 ovalbumin and formic acid showing a functionality of 131.2% compared to the original AAD. However,
372 isopropanol combined with other proteins results in significantly lower functionalities. Denatured
373 ethanol together with formic acid seems to give the most robust functionalities: combined with all 3
374 proteins, it shows a functionality of about 110% compared to the original AAD.
375
376 Even if more functional and greener, it is not desirable that the price of the AAD increases too much.
377 Keeping the AAD economically advantageous is next to ecological optimization and functionality an
378 important factor in the bigger picture of sustainability. A price estimation per batch of 150 mL was made
379 based on the current prices of the used products in the EU, acknowledging that these prices may vary in
380 time and per region.

381 No significant differences in the cost of the AADs can be observed, apart from the AADs containing
382 denatured ethanol in combination with ovalbumin and lactalbumin for which the costs are lower.

383
384 When combining the 3 responses in the overall desirability, it can be observed that the conditions using
385 denatured ethanol are the most desirable conditions in all 3 evaluated aspects (**Table 10** and **Figure 1**).
386 The conditions that use isopropanol and acetonitrile in combination with formic acid are respectively
387 less optimal and the least optimal choices compared to denatured ethanol. The choice of protein and acid

seem to be of less influence on the total desirability of the different AADs with exception of condition 11, which has a D value of 0 because of its lack of functionality.

The analysis model described in this article is not only useful for the evaluation of the AAD, but could easily be expanded to other analytical reagents as the production is very similar, i.e. weighing of a certain substance after which it is dissolved in a specific solvent with a defined composition (*e.g.* pH and organic solvent). However, this approach is unique in the sense that it evaluates the production of a reagent used in lab-scale analytical chemistry, despite the fact that reagents are quite common, often used and prepared, in chemical or pharmaceutical laboratories. The Ph.Eur. for example has currently around 3 000 reagents described (ref: Ph.Eur. 11.0 edition), with no attention paid to the different aspects as given in the balance discussed in this study. Additionally, in chemical and pharmaceutical Life Cycle Assessments (LCA), hardly any attention is given to the production of analytical reagents (or even analytics in general*).*

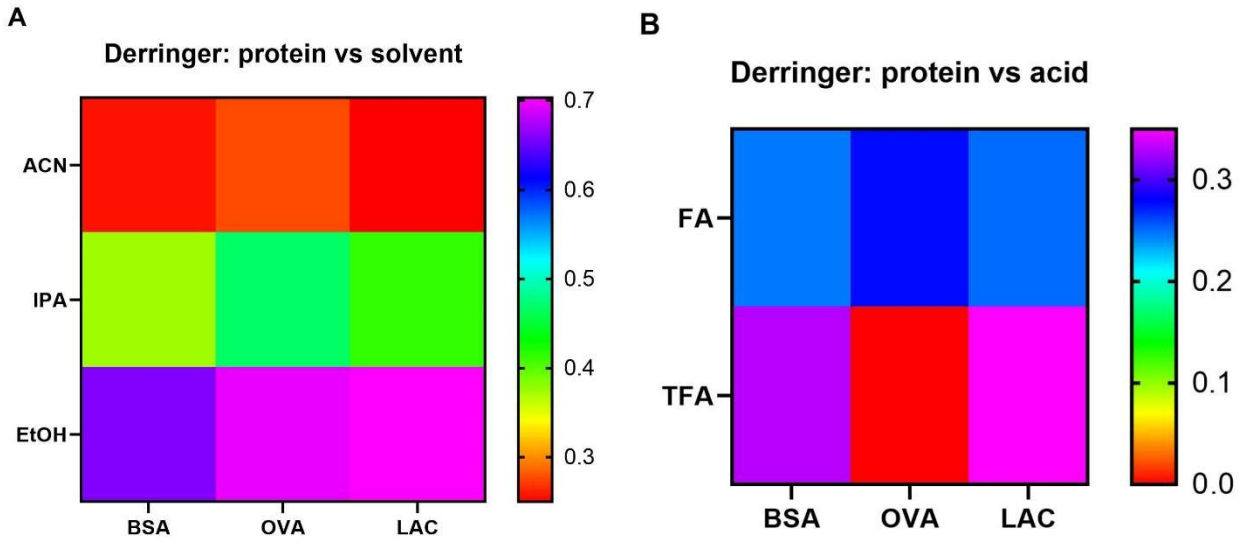

Figure 1: Derringer desirability results in function of A) protein and solvent and B) protein and acid. The conditions in panel B use ACN as a solvent.

4. Conclusion

We have evaluated a total of 17 AAD compositions on their functionality, greenness and cost by use of a Derringer desirability function. These compositions differed in organic solvent, protein and/or acid used. The change of solvent was responsible for the largest variation in desirability. The best alternative

choice would be one of the AAD conditions using denatured ethanol, which are the most optimal alternatives in all 3 evaluated aspects.

5. Acknowledgements

This work was supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (131356 to FV).

6. References

American Chemical Society - Solvent Selection Tool [Internet]. 2018. Available from: https://www.acs.org/content/acs/en/greenchemistry/research-innovation/tools-for-green-chemistry/solvent-selection-tool.html

Anastas, P., Warner, J., 1998. Green chemistry: theory and practice. Oxford University Press, Oxford.

Claux, O., Santerre, C., Abert-Vian, M., Touboul, D., Vallet, N., Chemat, F., 2021. Alternative and sustainable solvents for green analytical chemistry. Current Opinion in Green and Sustainable Chemistry. 31, 100510. https://doi: 10.1016/j.cogsc.2021.100510.

Derringer, G., Suich, R., 1980. Simultaneous Optimization of Several Response Variables. J. Qual. Technol. 12, 214–219. https://doi: 10.1080/00224065.1980.11980968.

Dhoru, M., Shah, K., Detholia, K., Patel, M., 2020. Green chromatography: the eco-friendly and safer concept of green analytical chemistry. Int. J. Pharm. Sci. Res. 11, 1022. https://doi: 10.13040/IJPSR.0975-8232.11(3).1022-32.

Dogan, A., Tobiszewski, M., 2020. Optimization of liquid chromatographic separation of pharmaceuticals within green analytical chemistry framework. Microchem. J. 152, 104323. https://doi:10.1016/j.microc.2019.104323.

Donohue, M. J., Filla, R. T., Steyer, D. J., Eaton, W. J., Roper, M. G., 2021. Rapid liquid chromatography-mass spectrometry quantitation of glucose-regulating hormones from human islets of Langerhans. J. Chromatogr. A. 1637, 461805. https//doi:10.1016/j.chroma.2020.461805.

Dunning C. Where Did My Sample Go? [Internet]. 2019. Available from: https://blog.waters.com/where-did-my-sample-go.

European Union, “Regulation (EC) no 1272/2008 of the european parliament and of the council of 16 December 2008.” 2008.

Fouda, H., Nocerini, M., Schneider, R., Gedutis, C., 1991. Quantitative analysis by high-performance

liquid chromatography atmospheric pressure chemical ionization mass spectrometry: the determination of the renin inhibitor CP-80,794 in human serum. J. Am. Soc. Mass Spectrom. 2, 164–167. https://doi.org/10.1016/1044-0305(91)80010-5.

Gałuszka, A., Migaszewski, Z. M., Konieczka, P., Namieśnik, J., 2012. Analytical Eco-Scale for assessing the greenness of analytical procedures. TrAC - Trends in Analytical Chemistry. 37, 61–72. https://doi: 10.1016/j.trac.2012.03.013.

Gałuszka, A., Migaszewski, Z., and Namieśnik, J., 2013. The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. TrAC - Trends in Analytical Chemistry. 50. https://doi: 10.1016/j.trac.2013.04.010.

Gionfriddo, E., 2020. Green analytical solutions for sample preparation: Solid phase microextraction and related techniques. 5, 20200006. https://doi: 10.1515/psr-2020-0006.

Goebel-Stengel, M., Stengel, A., Taché, Y., Reeve, J. R., 2011. The importance of using the optimal plasticware and glassware in studies involving peptides. Anal. Biochem. 414, 38–46. https://doi: 10.1016/j.ab.2011.02.009.

Jimenez-Gonzalez, C., Ponder, C. S., Broxterman, Q. B., Manley, J. B., 2011. Using the right green yardstick: Why process mass intensity is used in the pharmaceutical industry to drive more sustainable processes. Org. Process Res. Dev. 15, 912–917. https://doi: 10.1021/op200097d.

Jung M. Lost Samples in the Container: Non-specific Binding and the Impact of Blocking Agents [Internet]. 2019. Available from: https://blog.waters.com/lost-samples-in-the-container-non-specific-binding-and-the-impact-of-blocking-agents.

Michalski, R., Pecyna-Utylska, P., 2020. Ion chromatography as a part of green analytical chemistry. Arch. Environ. Prot., 46. https://doi: 10.24425/aep.2020.135759.

Nowak, P. M., Wietecha-Posłuszny, R., Pawliszyn, J., 2021. White Analytical Chemistry: An approach to reconcile the principles of Green Analytical Chemistry and functionality. TrAC - Trends in Analytical Chemistry. 138, 116223. https://doi: 10.1016/j.trac.2021.116223.

Pena-Pereira, F., Wojnowski, W., Tobiszewski, M., 2020. AGREE - Analytical GREEnness Metric Approach and Software. Anal. Chem. 92, 10076–10082. https://doi: 10.1021/acs.analchem.0c01887.

Płotka-Wasylka, J., 2018. A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index. Talanta. 181, 204–209. https://doi: 10.1016/j.talanta.2018.01.013.

Płotka-Wasylka, J., Mohamed, H. M., Kurowska-Susdorf, A., Dewani, R., Fares, M. Y., Andruch V., 2021. Green analytical chemistry as an integral part of sustainable education development. Current Opinion in Green and Sustainable Chemistry. 31, 100508. https://doi:10.1016/j.cogsc.2021.100508.

Prat, D., Hayler, J., Wells, A., 2014. A survey of solvent selection guides. Green Chem.. 16, 4546–4551. https://doi: 10.1039/c4gc01149j.

Prat D., Wells, A., Hayler, J., Sneddon, H., McElroy, C., Abou-Shehada, S., Dunn, P., 2015. CHEM21 selection guide of classical- and less classical-solvents. Green Chem. 18, 288–296. https://doi: 10.1039/c5gc01008j.

Ribeiro, M. G. T. C., Costa, D. A., Machado, A. A. S. C., 2010. 'Green Star': A holistic green chemistry metric for evaluation of teaching laboratory experiments. Green Chem. Lett. Rev. 3, 149–159. https://doi: 10.1080/17518251003623376.

Sajid, M., Płotka-Wasylka, J., 2022. Green analytical chemistry metrics: A review. Talanta. 238, 123046. https://doi: 10.1016/j.talanta.2021.123046.

Sheldon, R. A., Brady, D., 2022. Green Chemistry, Biocatalysis, and the Chemical Industry of the Future. ChemSusChem. e202102628. https://doi: 10.1002/cssc.202102628.

Van Dorpe, S., Adriaens, A., Vermeire, S., Polis, I., Peremans K., De Spiegeleer, B., 2011. Desirability function combining metabolic stability and functionality of peptides. J. Pept. Sci. 17, 398–404. https://doi: 10.1002/psc.1323.

Verbeke, F., Bracke, N., Debunne, N., Wynendaele, E., De Spiegeleer B., 2020. LC-MS Compatible

490 Antiadsorption Diluent for Peptide Analysis. Anal. Chem. 92, 1712–1719; https://doi:
491 10.1021/acs.analchem.9b01840.