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Replace, reduce, and reuse organic solvents in peptide downstream processing: the benefits of dimethyl carbonate over acetonitrile[†]

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Preparative liquid chromatography under reversed phase conditions (RPLC) is the most common method for the purification of therapeutic peptides in industrial downstream processing. Routine methods typically involve the use of aqueous buffers and acetonitrile (ACN) as the organic modifier. This choice is driven by the unique chemico-physical properties of ACN. However, ACN poses health and environmental risks, which downgrades it among the preferred choices from the greenness point of view. In this work, a step towards the greening of the downstream purification process is presented for three polypeptides following the three Rs of green analytical chemistry: replace, reduce, and reuse. This is achieved by using a mixture of isopropanol (IPA) and dimethyl carbonate (DMC) as green alternative solvents to ACN. A DMC/IPA mixture allows the reduction of the toxicological impact and amount of waste generated while preserving comparable process performance. Moreover, the possibilities of distilling and reusing the solvent waste derived from the chromatographic operations and lyophilizing the peptide solution without affecting the finite product quality have been explored.

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1. This work demonstrates for the first time the applicability of dimethyl carbonate as a green alternative solvent to acetonitrile for the purification of three industrial crude mixtures of polypeptides with up to 32 amino acids.
2. The use of dimethyl carbonate, alone or in a mixture with isopropanol, permits achieving comparable purification performance, in terms of purity and recovery of the target peptide, but with a reduced amount of organic solvent waste, increasing the greenness and the sustainability of the process. Moreover, the solvent waste derived from the chromatographic operations can be distilled and reused for subsequent purifications without affecting product quality.
3. The single-column purification process described can be made greener using multicolumn chromatography, permitting an increase in productivity while reducing solvent consumption. This process can also be scaled up to industrial scale separations.

1. Introduction

The area of therapeutic peptides has gained incredible attention from academia and industry in recent times. In fact, the

recent recognition of obesity as a serious pathology has led to an astounding increase in clinical trials of new peptide-based therapeutic candidates that are able to regulate body weight and to the shortage of already marketed drugs like the blockbuster semaglutide (Ozempic/Wegovy) and tirzepatide (Monjaro/Zepbound).¹

From a chemical point of view, the new wave of therapeutic peptides follows a harmonized strategy: an amino acid sequence length in the range of 30 to 40 amino acids and several non-proteinogenic amino acids added to the primary structure to improve the physicochemical properties.^{2,3} A remarkable example of the latter feature is the lipidated-PEGylated side chain present in the semaglutide and tirzepatide sequences, which are able to extend their blood bioavailability over one week.^{4,5} As a natural consequence of this trend, the manufacturing methodologies are increasingly

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preferring the chemical approaches over the fermentation ones due to their flexibility in inserting such non-canonical amino acid residues.

However, chemical approaches are usually associated with complex crude peptides with several unwanted impurities related to the selected chemical route.^{6,7} These impurities can be easily removed if they are process-related (*e.g.* scavenger, protecting groups, reagents) but become cumbersome in the case of product related impurities (*e.g.* diastereomers, aspartimides, deamidated sequences and aggregates). This complexity is further exacerbated by the recent guidelines issued by the regulatory agencies regarding the quality attributes for generic peptides. Indeed, in the case of the US Food and Drug Administration (FDA), specifications of <0.10% for each unknown impurity and $\leq 0.5\%$ for the identified ones have been imposed⁸ together with a limit on their residual solvent.⁹ These features altogether require the development of complex and extensive downstream processes in order to achieve the desired target of purity, where preparative liquid chromatography (prep-LC) is in most cases the only option available. The great advantages of the prep-LC technique rely on its versatility and scalability according to the geometrical diameter of the column.¹⁰

On the other side, this technology belongs to the dilution techniques, where the purified product is usually eluted at a lower concentration with respect to the concentration injected, leading to the possible adoption of a subsequent concentration step (*e.g.* rotary evaporation or tangential flow filtration)¹¹ and the final lyophilization or spray-drying step to recover the desired product as a powder. Furthermore, prep-LC requires a large volume of solvent to be fed to the column to allow the proper elution and purification of the target product. The fate of this mobile phase, which is usually a mixture of an aqueous buffer and organic solvent plus additives, is that it has to be either recycled or discarded. Taking into consideration that a chromatographic protocol usually requires several hundred litres of solvents to purify 1 kg of peptide (according to the feed complexity)¹²⁻¹⁴ and that a remarkable percentage of this volume (10% up to 40% according to the nature of the target peptide) is constituted by the organic modifier, the amount of potential waste becomes extremely relevant, with a negative impact on the environmental footprint of the purification processing.

However, not only the amount of waste produced but also its nature can represent an environmental issue. To classify the solvents based on their degree of health and environmental risks, several pharmaceutical companies have created their “solvent selection guide”, where the substances are organized into three groups (preferred, acceptable, and undesirable).¹⁵⁻¹⁷ Usually, the main organic solvent used in reversed-phase applications is acetonitrile (ACN). ACN is chosen thanks to its low viscosity, UV transparency, low reactivity, and relatively low boiling point (which facilitates the removal after the purification); but at the same time, it is associated with several drawbacks such as shortages, chemical instability and potential toxicity for both the environment and

human health, especially after hepatic metabolism, which catalyses the formation of cyanide.¹⁸ This latter aspect is reflected in the permitted limit of no more than (NMT) 410 ppm in the finite pharmaceutical ingredient, as imposed by the International Conference of Harmonization (ICH) guidelines.⁹

Greening the processes for the pharmaceutical industries can be achieved by applying the so-called three Rs: reduce, replace and recycle: the reduction of the waste produced, the replacement with greener materials (*e.g.*, solvents and additives), and the recycling or reuse of the solvent(s).^{19,20} By minimizing waste, industries can significantly lower their environmental impact, enhancing sustainability and efficiency. Additionally, the use of eco-friendly solvents, which are less harmful to both human health and the environment, ensures that chemical processes are cleaner and safer. In addition, the possible recycling of waste (*e.g.*, distilling the solvent) can help maximize the use and reduce the need for fresh resources. Together, these strategies represent a robust approach for achieving greener industrial practices, according to the principles of circular and green analytical chemistry (GAC).^{21,22} Some points of GAC related to the toxicity of the resources used are gaining more and more importance, including:

- GAC 7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.

- GAC 10. Reagents obtained from renewable sources should be preferred.

- GAC 11. Toxic reagents should be eliminated or replaced.

- GAC 12. The safety of the operator should be increased.

Very few alternatives are currently available as organic modifiers to replace ACN, with low boiling point alcohols as unique candidates (*e.g.*, methanol, ethanol, isopropanol).^{23,24} However, these solvents show comparable or lower elution strength with respect to ACN (thus similar or higher volumes are required to elute the desired peptide). An exception is represented by IPA, which is a stronger organic modifier than ACN under reversed-phase conditions and, for this reason, it is widely used at the industrial level. Some of these alcohols might possess intrinsic reactivity towards peptides such as in the esterification of the free carboxylic functions.²⁵ This is the case especially for methanol, as demonstrated in some studies conducted as part of this work and reported in the ESI (see section 3.2 and S6†).

To overcome these issues, in two recent companion papers, we have introduced the application of dimethyl carbonate (DMC) in both analytical and semi-preparative chromatography as a sustainable alternative to ACN.^{26,27} These studies highlighted that the elution strength of DMC is roughly 3 times higher with respect to ACN, thanks to which a less amount of organic modifier is needed to adequately elute both small molecules and a target peptide. Particularly, this green solvent was used to purify a mixture of icatibant, which already had a high level of purity due to its small size (only 10 amino acids).

In the present article, the three Rs have been applied to the purification of three polypeptides. In more detail, DMC is used



as an alternative to ACN (replace) for the first time and as an organic modifier to purify industrial feeds of large polypeptides (up to 32 amino acids) that contain a high number of impurities (*e.g.*, initial purity was 37% for semaglutide feed). In this case, given the poor miscibility of DMC with water (only up to 10% v/v), its elution strength is limited.²⁸ Hence, isopropanol (IPA) was added as a cosolvent to the mobile phase to successfully promote the elution of larger peptides, as demonstrated by the retention curves built under analytical conditions. Moreover, the addition of IPA in the mobile phase (15% v/v) increases the miscibility of DMC with water up to 15%. This mixture, which contains 30% of organic solvent (15% IPA + 15% DMC), shows a much higher elution strength than 30% ACN, meaning that a smaller organic content is sufficient to obtain the same retention behaviour for a given molecule (reduce). This also implies that the waste collected at the outlet of the chromatographic platform contains a smaller organic amount which is, in addition, more easily manageable than ACN.

To assess the impact of this approach with respect to the use of ACN, different metrics have been adopted, such as calculation of the *E*-factor and “Chemical Hazard Evaluation for Management Strategies” (CHEMS-1). The *E*-factor is a mass-based green metric which measures the amount of waste generated in a chemical process (*e.g.*, byproducts, leftover reagents, solvent losses, *etc.*) per mass of product produced or purified. Its calculation depends on the definition of waste, often excluding harmless substances like water.²⁹ On the other hand, CHEMS-1 is a chemical ranking and scoring method that also takes into account the hazard of chemicals to humans, animals and the environment.³⁰

Besides discussing the application of DMC in the downstream processes of commercially relevant therapeutic peptides, their subsequent post-purification steps have also been investigated. Indeed, the possibility to distil and reuse the exhaust solvent derived from chromatographic operations (recycle) and to freeze-dry the purified pool without compromising the final product quality has been considered. This work demonstrates the feasibility of DMC as a fully green alternative solvent to ACN for peptide purification, despite its scarce solubility in water which nevertheless can be overcome by adding an alcohol as a cosolvent.

2. Experimental

2.1 Reagents

Acetonitrile (ACN), ethanol (EtOH) and isopropanol (IPA) were purchased from Carlo Erba Reagents (Rodano, Milano, Italy) and all other reagents for buffers were purchased from Merck-Sigma Aldrich (St Louis, MI, USA). Dimethyl carbonate (DMC) was purchased from Thermo Scientific (Waltham, Massachusetts, USA).

Trifluoroacetic acid (TFA), acetic acid (AcOH) and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma Aldrich (Missouri, USA). Uracil, used to determine the

hold-up volume of the columns, was purchased from Sigma Aldrich (Missouri, USA).

2.2 Sample preparation

The three peptides considered in this work are degarelix, semaglutide and salmon calcitonin, made of 10, 31 and 32 amino acids, respectively. Degarelix, semaglutide and salmon calcitonin crude mixtures, together with the pure peptides used for the retention and the calibration curves of the analytical method were manufactured and provided by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy).

Feed solutions were prepared to reach final concentrations in the range of 8–10 g L⁻¹. The feed preparation protocols are described in ESI section S1.†

2.3 Retention curves

To investigate the retention behavior of the three peptides with both ACN and DMC, isocratic runs were performed under analytical conditions by employing an Agilent 1290 Infinity UHPLC (Agilent, Santa Clara, CA, USA), equipped with a binary solvent pump, a diode array detector (DAD) set at 220 nm and an autosampler. The temperature was set at 25 °C. The columns employed for the retention study of all the peptides were the Daisogel-SP-120-10-C8-BIO by Dr Maisch for semaglutide and calcitonin, and a Daisogel-SP-120-10-ODS-BIO by Dr Maisch for degarelix, both with dimensions of 250 × 4.6 mm with a column volume (CV) of 4.15 mL, pore size of 120 Å and particle size of 10 µm. The same columns were also used for the purification of peptides under preparative conditions, as described later. Injection volume was 5 µL, while the flow rate was set to 1.5 mL min⁻¹. The retention curves have been obtained by using mobile phases containing different additives, as reported in Table 1. Retention factors of the three peptides were measured at different fractions of the organic modifier (φ), directly obtained by binary solvent mixing (MP-A and MP-B) of the HPLC instrument. φ represents the sum of the fractions of both DMC and IPA (*e.g.*, 15% DMC + 15% IPA = 30% organic solvent, so φ = 0.3).

2.4 Purification of peptide mixtures

For all the peptides, purifications were performed on an ÄKTA pure 25L instrument (Cytiva/GE Healthcare, Uppsala, Sweden), operated through the Unicorn software. The instrument was equipped with a fraction collector. The detector wavelength was set at 300 nm. The columns were the same as already described in section 2.3. For each peptide, the purification was performed using ACN and DMC + IPA alternatively as organic modifiers. In every case, both MP-A and MP-B contained a given amount of aqueous buffer and of organic modifier. This choice was made in order to improve the mixing of the aqueous and organic phases during the gradient.

The details of all the purification methods are listed in section S3 of the ESI.† All methods included a loading step (where the feed is loaded into the column with a low percentage of organic modifier), an isocratic column equilibration step, two gradient steps to progressively increase the elution



Table 1 Mobile phase (MP) compositions (A and B) used to study retention of the three peptides

Peptide	Conditions with ACN		Conditions with DMC	
	MP-A	MP-B	MP-A	MP-B
Degarelix	1 $\text{H}_2\text{O} + 1\% \text{AcOH}$ (pH = 2.5)	ACN + 1% AcOH	$\text{H}_2\text{O} + 1\% \text{AcOH}$ (pH = 2.5)	MP-A/DMC/IPA 70/15/15 (pH = 2.7)
	2 $\text{H}_2\text{O} + 0.05\% \text{TFA}$ (pH = 1.9)	ACN + 0.05% TFA	$\text{H}_2\text{O} + 0.05\% \text{TFA}$ (pH = 1.9)	MP-A/DMC/IPA 70/15/15 (pH = 1.9)
Semaglutide	1 $\text{H}_2\text{O} + 40 \text{ mM } \text{NH}_4\text{HCO}_3$ (pH = 8.7)	ACN + 40 mM NH_4HCO_3	$\text{H}_2\text{O} + 40 \text{ mM } \text{NH}_4\text{HCO}_3$ (pH = 8.7)	MP-A/DMC/IPA 70/15/15 (pH = 8.9)
	2 $\text{H}_2\text{O} + 0.05\% \text{TFA}$ (pH = 1.9)	ACN + 0.05% TFA	$\text{H}_2\text{O} + 0.05\% \text{TFA}$ (pH = 1.9)	MP-A/DMC/IPA 70/15/15 (pH = 1.9)
Salmon calcitonin	1 $\text{H}_2\text{O} + 0.05\% \text{TFA}$ (pH = 1.9)	ACN + 0.05% TFA	$\text{H}_2\text{O} + 0.05\% \text{TFA}$ (pH = 1.9)	MP-A/DMC/IPA 70/15/15 (pH = 1.9)

strength of the mobile phase, and a final stripping step to clean and regenerate the column. During the gradient elution, fractions were collected and analyzed offline according to the analytical method chosen for every peptide, to gain information on the recovery and purity of the target peptide within a given pool.

2.5 Analytical method

For the offline analysis of all collected fractions, an Agilent 1290 Infinity UHPLC system (Agilent, Santa Clara, CA, USA) was employed. The detailed analytical methods for the three peptides are reported in ESI section S4.†

Diluted standard solutions of the three peptides used for the calibration curves were prepared by dissolving the pure peptide in the same dissolution solvent as described for the crude mixture, in a concentration range from 0.1 to 3 g L⁻¹. The calibration curves were then employed to determine the concentration of the peptide of interest in each fraction collected.

2.6. Post-purification steps (stability of peptides in DMC and lyophilization)

To evaluate the post purification step performances, we selected semaglutide as the case study, due to its structural complexity and its current market position. The isolation step of the purified semaglutide pooled fraction was performed following two different approaches:

(1) In the first one, 50 ml of purified semaglutide solution was subjected to evaporation of the organic solvent under vacuum with the aid of a rotavapor, keeping the water bath at a temperature of 25 °C. The obtained concentrated solution was lyophilized according to the following recipe: a step of sample loading followed by two freezing steps (a ramp for 4.5 h from 20 to -40 °C and a hold step for 1.5 h at -40 °C) and two drying steps (a ramp from -40 °C to 45 °C at 0.1 mbar for 15 h and a hold step at 45 °C and 0.1 mbar for 43 h).

(2) In the second approach, 50 ml of the purified semaglutide solution was directly lyophilized according to the same procedure used in the first approach, skipping the step of the rotavapor.

The amount of DMC and IPA left in the lyophilized powder was determined through gas chromatography using a GC Agilent 7820A, equipped with a split/splitless injector, sup-

ports for semi-capillary columns, and FID detector, with the autosampler HS Agilent 7697A.

2.7. Distillation of solutions containing DMC/IPA and ACN

Distillation was performed to evaluate the possibility to recover DMC from the solvent waste produced during the chromatographic purifications. The column distillation system consisted of an oil-bath heater to heat the solution, a Vigreux distillation column equipped with a temperature probe, a condenser, and a receiver where the distillate was collected.

Two solutions (1 L each) were prepared to evaluate the evaporative properties of both ACN and DMC aqueous mixture. The first solution contained 70/15/15 H₂O/DMC/IPA (same composition as the mobile phases used for the purification of peptides), whereas a second solution containing the same total organic content (30%) was prepared by mixing 70/30 H₂O/ACN. Distillation was performed by monitoring the distillation temperature at ambient pressure until complete evaporation of the organic solvent.

To achieve this, the distillate was sampled every 50 mL to analyze the content of IPA and DMC by means of the same GC-FID already cited. Frequent sampling allows for monitoring of the composition of the distillate throughout the entire distillation process. Additionally, the analysis of the residual undistilled solution enables verification of whether the entire organic fraction has been recovered. The distilled solution (containing also a small percentage of water), after an accurate dilution with the proper aqueous buffer in order to reconstitute the MP-B composition, was then tested for the purification of semaglutide, as described in section 2.4.

3. Results and discussion

3.1 Retention studies and purification performance

This study began with the investigation of the retention behaviour of the three peptides by changing the MP composition under isocratic conditions. The MP was a solution of aqueous buffers modified with variable percentages of organic modifier (see Table 1). It was known from our previous studies^{26,27} that DMC is only around 10% miscible with water, but its solubility increases if a cosolvent (e.g., low boiling point alcohol) is added to the mobile phase. This also contributes to improving the elution strength of the mobile phase, an aspect which is of



utmost importance for large peptides. To do so, a determined amount of IPA was added to the mobile phase. The solution was shaken and sonicated with ultrasound until the complete removal of the bubbles. IPA was chosen since it is one of the alcohols with the highest elution strength in LC and lower reactivity and toxicity profiles.¹³ Particularly, 15% IPA was added to the solution, and this amount favoured an increase in miscibility of DMC in water up to 15%. For the retention curves, the proportion of aqueous and organic solvents in MP-B was 70/15/15 water/DMC/IPA. This resulted in a maximum of 30% organic fraction, a composition able to elute all the peptides considered in this work at V_0 (hold-up volume of the column), as shown here.

The curves were built with both ACN and the mixture of DMC/IPA as modifiers, to make a comparison between the traditional and the green solvents. Besides comparing the organic solvents, two different widely used additives in the aqueous phase were also tested for each peptide, namely 0.05% TFA (a counterion often used for analytical purposes) and the counterion used for preparative applications specific for each peptide (ammonium bicarbonate 40 mM for semaglutide; 1% acetic acid for degarelix). Calcitonin, in contrast, was purified with TFA, therefore its curves have been only built with this aqueous buffer (for the details on mobile phase compositions, please refer to Table 1).

The retention curves are presented as $\log k$ vs. ϕ plots in Fig. 1, k being the retention factor, defined as $k = (V_R - V_0)/V_0$, with V_R the retention volume. The trend is associated with the traditional reversed phase behaviour, where the logarithm of retention decreases linearly when increasing the organic solvent amount in the mobile phase. Also, as expected, the larger the peptide, the higher the retention for the same ϕ and the steeper the curve. Steep slopes indicate a more marked dependence of the retention on the amount of organic modifier, as it usually happens with large biomolecules.³¹

Concerning degarelix, both aqueous buffers tested had an acidic pH and, as expected, the slopes of the four curves were very similar, meaning that the acid chosen has no influence on the retention; moreover, it can be noted that TFA led to a larger retention with both organic modifiers when compared to AcOH. On the other hand, semaglutide is purified in basic conditions due to its slightly acidic isoelectric point (5.4). The retention of this peptide is very different when changing the pH of the aqueous buffer, due to a modification in the spatial conformation and in the charge distribution of the peptide. This finding is corroborated by the evidence that semaglutide is scarcely soluble below pH 6, while it is completely soluble above pH 6.³² This low solubility under acidic conditions could explain why it is more retained with TFA with respect to bicarbonate.

It was also evinced that the retention is much smaller when using the DMC/IPA mixture no matter the peptide. This is supported by our previous studies concerning this solvent, where it was demonstrated that the elution strength of DMC was nearly 3 times higher than that of ACN.²⁶ Also, the slopes of the retention curves obtained with DMC/IPA are a bit steeper than their

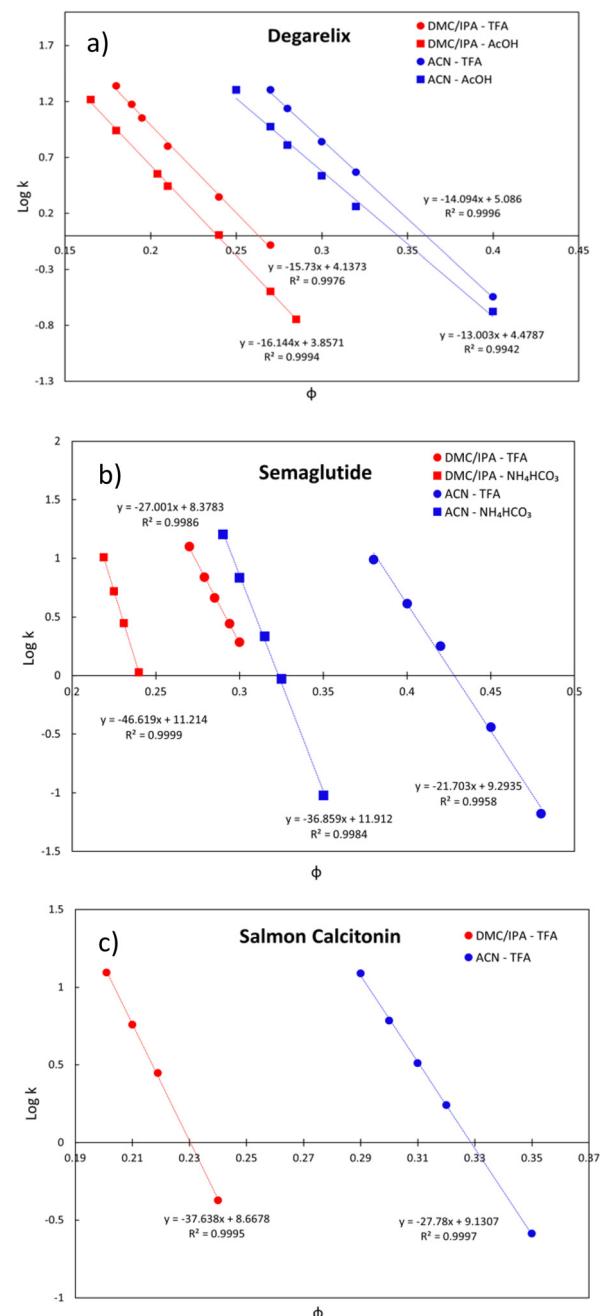


Fig. 1 Comparison of retention curves with ACN (blue) and DMC/IPA (red), with different additives, for each peptide. (a) Degarelix, (b) semaglutide, and (c) salmon calcitonin.

counterpart with ACN. This can be explained with the fact that a small change in the concentration of the organic modifier has a significant effect on the retention of biomolecules, this behaviour being more dramatic for strong solvents like DMC. In cases like these, it is recommended to work under gradient conditions during the purification processes, to modulate the separation of the target from impurities.

From this retention study, preparative LC methods for the purification of the three peptides were developed and opti-



mized by choosing a suitable range of φ , depending on the organic modifier (ACN, DMC or DMC/IPA). It was noted that, under preparative conditions, the peptides eluted at a lower organic percentage than under analytical conditions, probably due to a combination of the product–product interactions and the use of gradient. This feature was particularly evident in the case of degarelix where, despite not eluting from the column when using only DMC in MP-B (without IPA) under analytical conditions, its purification could be accomplished without the use of the alcohol as a cosolvent at the preparative scale. In this particular case, MP-B consisted of 89% aqueous buffer and 11% DMC.

In complex mixtures, the target product elutes between more weakly and more strongly adsorbed impurities. Therefore, to remove highly retained impurities from the column at the end of the purification method, a cleaning step involving a strong mobile phase is always required. In contrast, at the beginning of the run, the mobile phase must be weak enough to allow the binding of the analytes on the stationary phase. For these reasons, all products tested in our work were loaded with a low content of organic solvent. The purification strategy was performed through a two-step gradient, where the aim was to have the flattest slope as possible during the elution of the desired product. After the elution of the target peptide, a final cleaning step was performed in both cases using a high organic solvent concentration (up to 70%). In the case of DMC/IPA purification, a third mobile phase with a ratio of aqueous buffer/DMC/IPA 40/30/30 was used to standardize all the cleaning procedures for the three peptides (degarelix eluted in aqueous buffer/DMC only).

The methods have been developed to guarantee the same duration, for a given peptide, with the different organic solvents (e.g., total duration 10.75 CV, 13.25 CV and 14.25 CV for semaglutide, degarelix and calcitonin respectively, with both ACN and DMC or DMC/IPA). The duration of the flatter gradient (where the separation occurs) was kept constant for both solvents (see Fig. 2). Meanwhile, the cleaning step was kept constant in terms of time and organic concentration. Along the gradient, fractions were collected, and they were analyzed offline (see section 2.5 and section S4†) to evaluate the performance of each method in terms of purity, recovery, productivity and *E*-factor (see later on and section S2† for further information on the calculation of these parameters). Starting from these results, the so-called Pareto curves have been built. These plots, which display how purity decreases when increasing the recovery by enlarging the collection window, provide fundamental information about the outcome of a purification method.^{33–36} Pareto curves obtained with ACN and DMC/IPA are almost overlapped for the same peptide, indicating that green solvents are able to provide purification performance similar to the traditional ACN (Fig. 3).

Based on the fraction analysis reported in the Pareto curves (Fig. 3), the purest pool obtained with the two solvents were chosen; particularly, a requirement of 99.5%, 93.5 and 97% of purity has been set for degarelix, semaglutide and salmon calcitonin respectively. The different thresholds set for the pep-

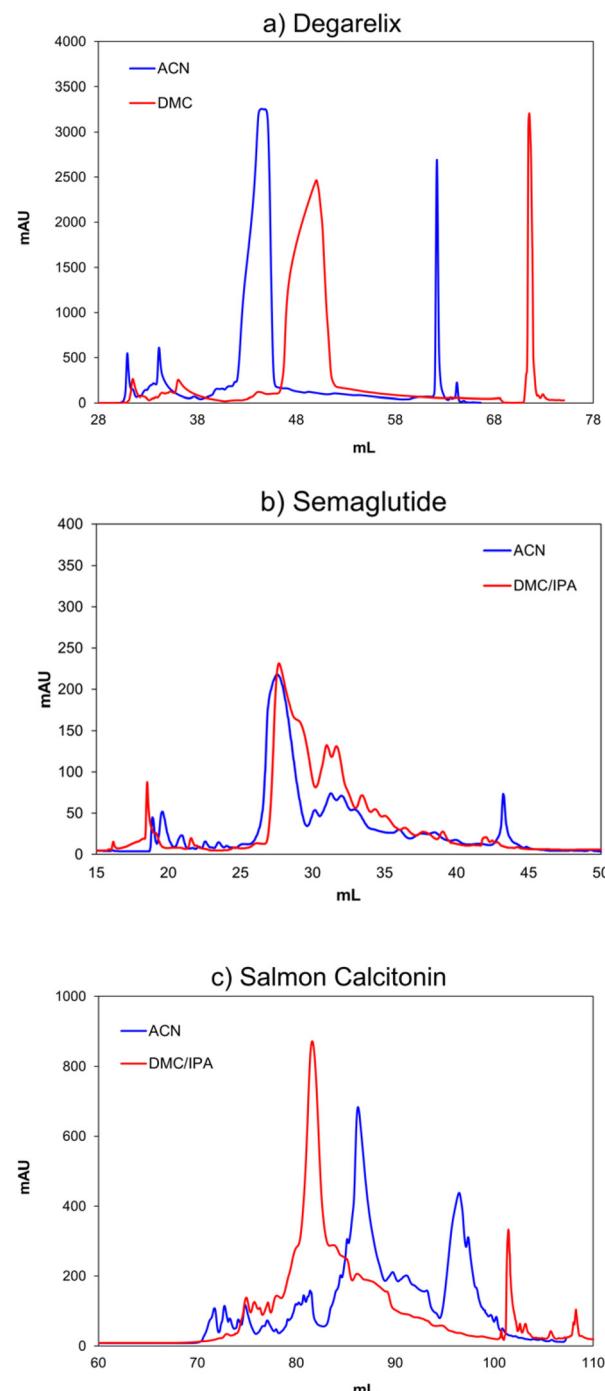


Fig. 2 Comparison of UV profiles obtained for each peptide with the preparative LC methods with ACN (blue line) and DMC/IPA (red line). (a) Degarelix, (b) semaglutide, and (c) salmon calcitonin.

tides reflect the differences in their initial purities. Also, it must be considered that these purification methods represent just part of the downstream processing of these peptides, which need to reach even higher purities before being used as active pharmaceutical ingredients (APIs).

The pools satisfying these requirements and having the highest recovery as possible have been compared for the two



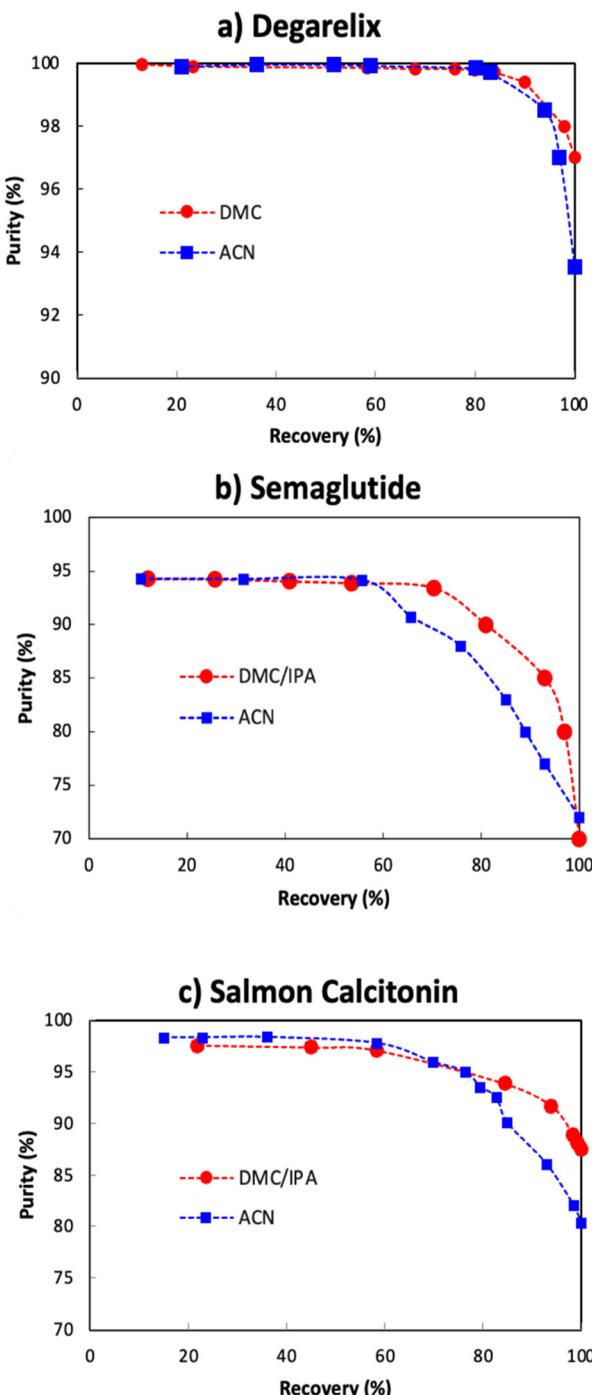


Fig. 3 Pareto curves obtained for the three peptides for DMC/IPA (red) and ACN (blue) as organic modifiers. (a) Degarelix, (b) semaglutide, and (c) salmon calcitonin.

solvents and the results are reported in Table 2. The data confirmed the comparable performance between the DMC/IPA mixture and ACN.

3.2 Stability of peptides in DMC/IPA vs. ACN and final isolation

The stability of the peptide pooled fractions after eluting from the column is critical for the proper set-up of a chromato-

graphic purification protocol. In most cases, the crude peptide coming from upstream cannot be purified in one single chromatographic run, due to the high batch size applied to increase productivity. Consequently, the pooled fractions coming from the first run have to wait for those coming from the last one before merging them all and achieving the final desired pool, and their purity must not be affected during this period. To verify this, a representative pool coming from semaglutide purified with the mixture of DMC/IPA and ACN was monitored over time at 2–8 °C. The percentages of organic solvents were obtained again from the elution time (see Table 3). The data confirmed that the concentration and the purity of all of them remain substantially constant within 10 days when stored at 2–8 °C, without a significant increase in impurity contents (see Table 3 for semaglutide and Tables S11 and 12† for salmon calcitonin and degarelix). On the other hand, a similar test was performed on semaglutide to verify its stability in a mixture of aqueous buffer and methanol (see Table S13†). Differently than DMC/IPA, in the case of methanol this peptide degrades and forms impurities attributable to its methyl esters derivatives, suggested by their *m/z* values of +14 found in the LC-MS experiments. Therefore, DMC proves to be a better choice with respect to methanol from the peptide stability point of view.

In all the cases discussed, the impurities are identified based on their relative retention time (RRT), meaning the ratio between their retention time and the retention time of the target compound. By doing so, possible shifts in the retention times are kept into account. The RRT of the target is therefore 1.00.

Lastly, the purified peptide pools reported in Table 2 have been isolated to verify the quality of the dried powder and the requirements necessary to remove DMC and IPA from the system. Two isolation protocols were tested: (1) one removing the organic solvent followed by the lyophilization of the obtained solution and (2) one lyophilizing the full mixture without removing the organic solvent. In both cases, the same lyophilization recipe was adopted, illustrated in section 2.6.

As expected, in the first case, the isolated powder showed a residue of both IPA and DMC below the limit of quantification (LOQ) assessed through gas chromatography (GC), confirming the capability of these solvents to be removed during the executed isolation protocol. In the second case, applying only the lyophilization step, the residual solvent content in the semaglutide powder was respectively below the quantification limit for IPA and 3879 ppm for DMC. Although the residual content of DMC might seem elevated, it actually remains within acceptable limits. In fact, the permitted daily exposure (PDE) for DMC has been quantified at 50 mg, corresponding to a concentration limit of 5000 ppm in the powder, assuming a daily intake of 10 g of the API. However, this intake is overestimated and exceeds by far the typical daily dose of the API in question. The derivation of these values is elucidated in section S9.†

This experiment confirmed the possibility of freezing the solution, allowing a proper lyophilization procedure even in the presence of these organic solvent mixtures, and the ability of

Table 2 Comparison of the performance of the purest pool for each method. Initial purity of each feed is also reported. *E*-Factor and pHV refer to metrics used to assess the greenness score of the purification methods (for further details refer to section 3.4)

Peptide	Initial purity (%)	ACN					DMC/IPA				
		Recovery (%)	Purity (%)	Productivity (g L ⁻¹ h ⁻¹)	<i>E</i> -Factor (L org g ⁻¹)	pHV	Recovery (%)	Purity (%)	Productivity (g L ⁻¹ h ⁻¹)	<i>E</i> -Factor (L org g ⁻¹)	pHV
Degarelix	84.44	80.23	99.84	4.02	0.66	580.22	76.02	99.81	3.81	0.38	93.46
Semaglutide	37.09	60.23	94.11	3.99	0.69	457.48	57.89	93.97	3.84	0.59	108.31
Calcitonin	45.53	58.44	97.75	3.15	0.79	505.18	58.37	97.11	3.15	0.70	129.14

Table 3 Stability of semaglutide dissolved in mixtures containing ACN (bottom) and DMC/IPA (top) at different time points. The purity (area%) of the target and the impurities are reported for different time steps, together with the percentage variation of the target concentration along time, expressed as the concentration of the peptide at a given time *t* with respect to the initial concentration

RRT	<i>t</i> = 0 day Area%	<i>t</i> = 2 days Area%	<i>t</i> = 10 days Area%
Semaglutide stability in NH ₄ HCO ₃ 40 mM/IPA/DMC (~76 : 12 : 12 v/v) <i>T</i> = 2–8 °C			
0.66	0.06	0.06	0.06
0.81	0.07	0.06	0.07
0.86	0.22	0.22	0.22
0.93	0.55	0.57	0.58
0.95	0.18	0.19	0.18
0.97	1.48	1.47	1.48
0.99	0.80	0.77	0.78
1.00	95.82	95.77	95.62
1.09	0.18	0.17	0.18
1.10	0.06	0.07	0.07
1.19	0.17	0.16	0.16
Conc. <i>t</i> /conc. <i>t</i> ₀	100%	100%	98.9%
Semaglutide stability in NH ₄ HCO ₃ 40 mM/ACN (~66 : 34 v/v) <i>T</i> = 2–8 °C			
0.66	0.06	0.06	0.06
0.81	0.06	0.06	0.07
0.86	0.22	0.21	0.22
0.93	0.56	0.58	0.58
0.95	0.19	0.19	0.19
0.97	1.50	1.49	1.49
0.99	0.79	0.77	0.77
1.00	95.74	95.68	95.56
1.09	0.17	0.17	0.17
1.10	0.07	0.06	0.07
1.19	0.16	0.16	0.16
Conc. <i>t</i> /conc. <i>t</i> ₀	100%	101.9%	98.9%

the process to efficiently remove them. Whether the data obtained on residual DMC in the isolated product are acceptable for the pharmaceutical industry is still a matter of debate. In fact, while the IPA residual solvent specification in the isolated product is present and set to NMT 5000 ppm (common limit for class 3 solvent), currently there are no imposed limitations in the ICHQ3D on residual DMC permitted in the drug substance. Anyhow, considering the studies currently present in the literature describing the safety profile of DMC^{37,38} its residual limit in the drug substance is expected to be higher with respect to ACN (NMT 410 ppm).⁹ Furthermore, the lyophilization recipe could be further improved to control the content of residual DMC in the isolated peptide, ensuring lower values.

3.3 Distillation

Recovery of the organic solvent used in the chromatographic procedures is another key point to significantly improve the environmental impact of the process. As a preliminary study, we tested the behaviour of DMC/IPA/aqueous buffer and ACN/aqueous buffer mixtures upon distillation. To have a direct comparison, an equal volume of mixtures was prepared with the same amount of total organic solvent concentration (15/15/70 DMC/IPA/aqueous buffer and 30/70 ACN/aqueous buffer) and the distillation operative conditions were kept the same for both the distillations. In the case of DMC/IPA/aqueous buffer, the distillate fractions, analyzed by GC to determine the content of DMC and IPA, showed an enrichment of the organic phase due to the higher vapor pressure of these solvents compared to water. This is in accordance with the boiling point of the different solvents used: IPA 82.3 °C, DMC 90.4 °C, ACN 81.6 °C, water 100 °C.²⁶ Analysis of the fractions collected every 50 mL demonstrates a greater evaporation of DMC, followed by an enrichment in IPA as the DMC is depleted from the distillation solution. During the evaporation of the organic solvent, the vapor temperature stabilizes around 77 °C and remains constant until the completion of the organic solvent distillation, at which point the temperature reaches 99 °C. GC analysis of the non-distilled solution confirms the complete recovery of the organic solvent (see Table 4).

The comparative distillation protocol performed with an ACN/aqueous buffer mixture showed similar vapor temperature and distillation time (see Table S14†), suggesting that both DMC/IPA/aqueous buffer and ACN/aqueous buffer recovery involves comparable energetic requirements.

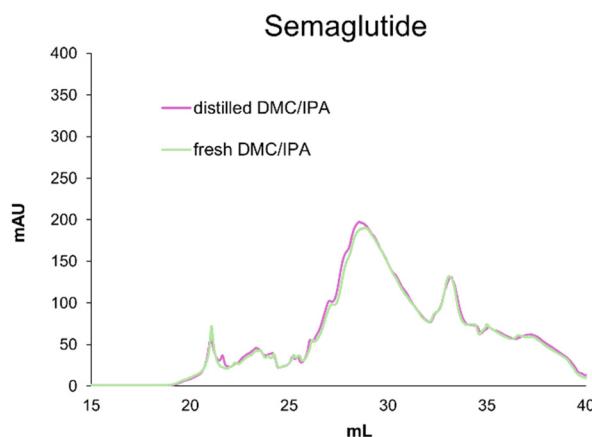
The performance of the recovered DMC/IPA mixture was tested on semaglutide. The recovered solvent fractions were merged and diluted with aqueous buffer in order to reproduce the same mobile phase composition (aqueous buffer/DMC/IPA in proportions 70/15/15 and 30/35/35, respectively). These were used to perform the purification of semaglutide, under the same chromatographic conditions as described in Table S5.† Even if small discrepancies in the chromatographic profiles were observed due to the use of different starting crude peptide, the UV profiles and composition of the fractions collected did not reveal any significant differences with respect to those obtained with mobile phases freshly prepared (see Fig. 4), and they are completely superimposable. This indicates



Table 4 Fraction analysis of a distilled solution of DMC/IPA/aqueous buffer 15/15/70 performed by GC

Distillation of 1000 mL of a solution of DMC/IPA/aqueous buffer 15/15/70

Por.	V (mL)	IPA (%)	DMC (%)	Total organic solvent (%)	Vapor temperature (°C)
1	50	26.22	62.47	88.69	77
2	100	27.52	61.39	88.91	
3	150	30.02	58.84	88.86	
4	200	34.28	55.00	89.28	
5	250	44.23	45.61	89.84	
6	300	68.60	11.76	80.36	77–98
7	350	3.41	0.09	3.50	99
8	400	0.27	0.21	0.48	
Undistilled	0.00	0.00	—	—	

**Fig. 4** Comparison of UV profiles obtained for the purification of semaglutide by using fresh (green) and distilled (pink) DMC-based mobile phases. Deviations between UV profiles in this figure and in Fig. 2B are due to the use of different crude mixtures.

that the solvent can be hypothetically recycled run after run and reused, without affecting the outcome of the purification, thus increasing the greenness of the process.

3.4 Metric comparison

The processes using green solvents (*i.e.* DMC or the mixture DMC/IPA) were compared to ACN in terms of green metrics. The first well-established parameter considered was the *E*-factor, which allows the comparison of the absolute consumption of organic solvents (thus not considering water). Accordingly, the purification protocols reported have been designed to maintain the same process duration (in terms of CV of the mobile phase employed), with the aim to focus on the consumption of the organic modifier and assess the overall *E*-factor. As expected, the comparison between *E*-factors obtained under the different conditions for the three peptides showed that this parameter is systematically lower for DMC/IPA than for ACN (−15%, −43% and −11% for semaglutide, degarelix and salmon calcitonin, respectively), thanks to the higher

elution strength of DMC/IPA with respect to ACN. On the other hand, recoveries and purities were comparable regardless of the solvents (see Table 2).

As a further comparison assessment, CHEMS-1 metric has been used. It is recognized by the European Chemicals Agency (ECHA)³⁹ (see section S8†) that both DMC and IPA show lower toxicity profile to humans compared to ACN, either for the acute or the chronic effect, disregarding the administration route. On the other hand, DMC is reported to have a higher impact on aquatic systems *versus* ACN. However, its higher eco-degradability than ACN, particularly with respect to hydrolysis susceptibility, mitigates the toxicological risk for the aquatic systems.³⁹ Furthermore, IPA shows lower toxicity than ACN also in this regard (see the ESI†). According to the calculations proposed by Tobiszewski,³⁰ the different parameters related to the hazard values (HV) have been considered for each solvent, with the aim to calculate the procedure hazard value (pHV). This parameter keeps also into account the volume of solvent employed for each method. The volume-weighted hazard values are a valuable tool for assessing the environmental impact of analytical methods, since they account for both the nature of the chemicals employed (toxicological risks for humans, animals and environment) and the experimental conditions used (namely, the chromatographic set-up). The results obtained for each solvent and for all the peptides are reported in Table 2 and show that, for all the peptides, the pHV is 70–80% lower than its counterpart with ACN. This is due to two reasons, namely the much smaller volumes of the organic modifier necessary for the DMC/IPA mixtures, due to the higher elution strength of DMC, and the lower hazard and toxicity of this mixture with respect to ACN.

For the reasons stated above, the DMC/IPA can be considered a desirable and lower impacting solution than ACN from the toxicological and environmental point of view.

4. Conclusions

The difficulty of choosing a substitute for acetonitrile in liquid chromatography is due to the struggle in finding other organic solvents offering comparable performance.²³ Starting from the previous work performed in our lab, this study further confirms that DMC can be a promising candidate for chromatographic purposes, especially for pharmaceutical applications, possibly in combination with IPA when a higher elution strength is required. The comparison with ACN for the purification of semaglutide, degarelix and salmon calcitonin highlights that similar levels of recovery are obtained for similar purities by using these two different organic modifiers but, on the other hand, DMC causes a decrease in the *E*-factor from 11% to 43% depending on the case. Moreover, the total hazard value, which takes into account different aspects of chemical volume and toxicity (pHV), shows a reduction of 70–80% for each peptide when using DMC/IPA. It was also demonstrated that it is possible to recover the DMC/IPA mixture from wastes generated during the preparative runs through distillation,



with comparable energy costs with respect to ACN. Furthermore, MPs prepared with recovered DMC and IPA lead to the same chromatographic profile obtained with freshly prepared ones. Using these peptides as reference products, it was confirmed by the stability studies that DMC and its mixtures do not react with the substrates either under acidic or basic conditions. Lastly, DMC (alone and in combination with IPA) was demonstrated to be efficiently removed during the standard isolation protocols (performed through lyophilization), ensuring a negligible residual amount in the obtained powder. Moreover, DMC/IPA can also be efficiently removed through the lyophilization step only, showing residual traces in the lyophilized pure peptide. This feature could become very important from the viewpoint of pharmaceutical companies since DMC is expected to have less strict limitations than ACN concerning its content in finite products.

Considering all this, this complete work underlines the possibility of using DMC as a green alternative to ACN in RP-LC, demonstrating that it allows attainment of similar performance to that of ACN with a lower amount of organic solvent required, thanks to its high elution strength. However, the limitation of its miscibility in water must always be taken into account, and this drawback can be overcome by adding an alcohol as a co-solvent. This combination expands its application to a broader class of peptides, maintaining high production performance and excellent environmental and health safety standards. Thus, employing DMC would help to move the pharmaceutical industry closer to the principles of Industry 4.0.²⁰

Author contributions

AR, AC, SF, WC, and MC have been responsible for funding acquisition, resources, conceptualization, supervision, and writing, reviewing and editing the paper. CDL, CN, MS, LM, GF, MC, and MM have been responsible for methodology, investigation, formal analysis, data collection and writing of the original draft. The manuscript was written in collaboration with all authors, who have given approval for the final version.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the ESI.†

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