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Dimethyl carbonate as a green alternative to acetonitrile in reversed-phase liquid chromatography. Part II: Purification of a therapeutic peptide

Desiree Bozza [a], Chiara De Luca [a], Simona Felletti [b], Matteo Spedicato [a], Francesco Presini [a], Pier Paolo Giovannini [a], Marco Carraro [c], Marco Macis [c], Alberto Cavazzini [a,d], Martina Catani [a,*], Antonio Ricci [c,*], Walter Cabri [c,e]

[a] Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, via L. Borsari 46, Ferrara 44121, Italy
[b] Department of Environmental and Prevention Sciences, University of Ferrara, via L. Borsari 46, Ferrara 44121, Italy
[c] Fresenius Kabi iPSUM, via San Leonardo 23, Villadose, Rovigo 45010, Italy
[d] Council for Agricultural Research and Economics, via della Navicella 2/4, Rome 00184, Italy
[e] Department of Chemistry "Giacomo Ciamician", Alma Mater Studiorum – University of Bologna, Via F. Selmi 2, Bologna, Italy

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ABSTRACT

Preparative liquid chromatography in reversed phase conditions (RPLC) is the most common approach adopted in the downstream processing for the purification of therapeutic peptides at industrial level. Due to the strict requirements on the quality imposed by the Regulatory Agencies, routinary methods based on the use of aqueous buffers and acetonitrile (ACN) as organic modifier are commonly used, where ACN is practically the only available choice for the purification of peptide derivatives. However, ACN is known to suffers of many shortcomings, such as drastic shortage in the market, high costs and, most importantly, it shows unwanted toxicity for human health and environment, which led it among the less environmentally friendly ones. For this reason, the selection of a suitable alternative becomes crucial for the sustainable downstream processing of peptides and biopharmaceuticals in general.

In this paper, a promising green solvent, namely dimethyl carbonate (DMC) has been used for the separation of a peptide not only in linear conditions but also for its purification through non-linear overloaded chromatography. The performance of the process has been compared to that achievable with the common method where ACN is used as organic modifier and to that obtained with two additional solvents (namely ethanol and isopropanol), already used as greener alternatives to ACN.

This proof-of-concept study showed that, thanks to its higher elution strength, DMC can be considered a green alternative to ACN, since it allows to reduce method duration while reaching good purities and recoveries. Indeed, at a target purity fixed to 98.5 %, DMC led to the best productivity with respect to all the other solvents tested, confirming its suitability as a sustainable alternative to ACN for the purification of complex biopharmaceutical products.

1. Introduction

Peptides are organic polymers composed by a series of amino acids (usually from 2 to 50), with molecular weight between 500 and 5000 Da. Many of them are called bioactive because they can have a beneficial impact on biological functions and, thus, on human health [1,2]. The research into this class of biomolecules has started at the beginning of the 20th century, especially by focusing on natural human hormones, including insulin, which has been synthetized for the first time in 1921. Reduced half-lives and poor oral availability have been the main challenges in peptide drug development, but recent advancements in synthetic strategies have allowed to produce novel candidates with better pharmacokinetics and specificity which have revamped the interest around this class of biomolecules. Today, more than 80 peptide-based drugs have been approved and commercialized, but almost 600 more candidates are under preclinical studies [3].

Among the many methods that can be employed to synthetize peptides, solid phase peptide synthesis (SPPS), introduced by Merrifield in

* Correspondig authors.
E-mail addresses: martina.catani@unife.it (M. Catani), Antonio.Ricci@fresenius-kabi.com (A. Ricci).

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early 60′s [4], is the most popular and widely used for industrial purposes, also thanks to the fact that it can be easily automated. Other methods involve the use of specific enzymes able to generate a peptide bond in water (chemoenzymatic synthesis) or recombinant technologies through an appropriate expression system [5]. However, none of these techniques allows to produce the target peptide alone and a series of unwanted impurities is also synthetized which has an impact on the purity of the final product. Among the impurities, process related ones (e.g., host cell proteins, viruses, etc.) can be easily removed; on the opposite, product related impurities (molecular variants of the target peptides, including fragments, diastereomers and aggregates) are much more challenging because they share common features with the target product [6,7]. For this reason, one or more purification steps are required to meet quality constraints for (bio)pharmaceuticals imposed by Regulatory Agencies before product commercialization.

Liquid chromatography (LC) in preparative conditions is routinary employed in industry for this purpose. This technique makes use of columns of higher dimensions with respect to common analytical LC and thus it requires the use of larger flow rates (in the order of 10–50 mL/min in preparative conditions up to several L/min in production scale chromatography) [8].

Peptides are commonly purified in reversed-phase conditions, by using hydrophobic stationary phases (C18 or C8, depending on the hydrophobicity of the peptide) and a mixture of aqueous solutions and an organic modifier as mobile phase. Since the retention of peptides is highly influenced by changes in organic modifier concentration [9,10], they are usually purified in gradient conditions, which are also mandatory to improve the resolution of the target peptide from its product-related impurities [11].

However, among all the techniques available for the purification of (bio)products, preparative chromatography is the highest demanding one in terms of energy and waste generation, due to the high dilution required to perform the process and the energy used to control and operate the gradient required for an efficient purification as well as the energy required to evaporate the solvent from the pool containing the purified product.

Consequently, several liters of aqueous and organic mixtures are generated daily as waste which needs to be disposed, with relevant costs and environmental impact, making the downstream process a critical bottleneck from the point of view of sustainability in the entire production process of a target (bio)pharmaceutical [5].

Furthermore, the most widely used organic modifier by (bio)pharmaceutical industries is acetonitrile (ACN), due to its excellent characteristics from a chromatographic point of view, including UV transparency. In particular, as of today, ACN is the only possible choice for the purification of therapeutical peptides at industrial level. However, this solvent suffers of severe shortcomings, including shortage periods linked to the industrial production of acrylonitrile, known as SOHIO process, where ACN is obtained as by-product of its process [12], and very high prices. Moreover, ACN is known to release cyanide upon metabolism in the hepatic cells, with all the drawbacks associated to the toxicity of this product, both from the human and the environmental point of view [13]. For this reason, the International Conference of Harmonization (ICH) guidelines classified ACN as Class 2 solvent with a residual allowed limit of not more than (NMT) 410 ppm in the pharmaceutical drugs [14]. The importance of the environmental and safety impact of the organic solvents is further demonstrated by the Green Chemistry roundtable established American Chemical Society, whose aim is to introduce the concepts of Green Chemistry into pharmaceutical manufacturing [15]. Even if, at the beginning, the focus was only on synthetic drugs, lately the attention has been moved also to biopharmaceuticals, drafting a detailed solvent selection guides which rank them according to their “greenness” impact [5,16–19].

In this optic, the search for alternative solvent with a lower impact from an environmental/toxicological point of view and with a broader availability in the market will become pivotal for ensuring a robust and sustainable downstream process.

In the companion paper, we have investigated the possibility of replacing ACN with dimethyl carbonate (DMC), one of the most promising emerging green solvents used for many industrial applications but barely applied to chromatography [20–26]. In that case, two small molecules were used to understand its retention properties from a fundamental viewpoint. In the comparison, also ethanol (EtOH) and isopropanol (IPA) were considered, since they are the most common green alternatives to ACN used as organic modifiers in LC [27].

This work is intended as a proof-of-concept study where DMC has been employed for the very first time in overloaded non-linear condition with the scope of verifying whether DMC can be effectively used as organic solvent, in replacement of ACN, for the purification of a pharmaceutically relevant peptide. To this end, an industrial perspective was adopted, where the goal is the comparison of the purification outcome in terms of process purity, recovery and productivity as a function of the solvent employed [10,28,29]. After comparing the retention behavior of the peptide using ACN, DMC and also EtOH and IPA, these solvents have been used for the isolation of the target product.

2. Theory

The purpose of preparative chromatography is the purification, isolation or accumulation of the target product; therefore, in contrast to analytical chromatography, preparative applications require large amount of sample to be processed [8,30]. The eluate is collected at the outlet in different fractions that are subsequently offline analyzed. Hence, chromatographic performance is estimated from analytical (ultra) high performance liquid chromatography ((U)HPLC), by quantifying four parameters: chromatographic purity, recovery, productivity, and solvent consumption. (Bio)pharmaceuticals must respect severe purity requirements, hence the first parameter to take into consideration is the chromatographic purity. It is obtained from the ratio of the target peak area (A_{target}) to the total area (A_{total}) of all the peaks present in the analytical chromatogram, which also includes impurities, and it is expressed as a percentage:

$$Purity\ (\%) = \frac{A_{target}}{A_{total}} \times 100 \tag{1}$$

Recovery is defined as the ratio of the mass of peptide contained in a fraction or pool of fractions ($m_{target\ collected}$) to the total peptide mass injected ($m_{target\ injected}$) into the system:

$$Recovery\ (\%) = \frac{m_{target\ collected}}{m_{target\ injected}} \times 100 \tag{2}$$

The productivity represents the ratio of amount of target peptide recovered in a fraction or a pool per unit of time and volume of stationary phase (which could be approximated with the geometrical volume of the column (CV)), which is calculated as the geometrical volume of the column:

$$Productivity\ (mg/L/h) = \frac{m_{pool\ collected}}{CV\ \times\ time} \tag{3}$$

Finally, the last parameter to take into account when determining the goodness of a purification process is solvent consumption, expressed as the ratio of the total MP volume used during the whole run and the mass of peptide contained in a fraction or pool. This parameter is important from the industrial point of view; indeed, it is convenient to achieve the lowest solvent consumption as possible. This parameter is defined as:

$$Solvent\ consumption\ (mL/mg) = \frac{Total\ Volume}{m_{target\ collected}} \tag{4}$$

3. Materials and methods

3.1. Sample preparation

The crude mixture (feed) of Icatibant, a cyclic peptide composed by ten amino acids was kindly given by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy) and it was obtained by means of solid-phase synthesis. The product content in the crude mixture was 43 % based on weight. A defined amount of feed was dissolved in 50 mM ammonium acetate/ACN solution, 97/3% v/v [28]. The final concentration of Icatibant was 2.5 g/L. Then, the solution was left under agitation for one hour and filtered with 0.20 μm filters prior to injection. For the purposes of this work (see further on), the feed (same concentration) was prepared also by employing either IPA, EtOH or DMC in place of ACN.

3.2. Non-linear overloaded chromatographic conditions

ACN, EtOH and IPA from Carlo Erba Reagents (Rodano, Milano, Italy) and all other reagents for buffers were from Merck-Sigma Aldrich (St. Louis, MI, USA). DMC (purity > 99 %) was from Thermo Scientific (Waltham, Massachusetts, USA).

Purifications were performed on a ÄKTA pure 25 L instrument (Cytiva/GE Healthcare, Uppsala, Sweden), equipped with a fraction collector, a detector set at 265 nm and operated through the Unicorn software. A 250 × 4.6 mm Daisogel-SP-120-10-ODS-BIO column, with a pore size of 120 Å and a particle size of 10 μm, was used for preparative runs; its geometrical volume was 4.15 mL. The feed prepared in Section 3.1 with a concentration of 10 mg/mL$_{column}$ was injected into the column by using a dedicated pump working at 3 mL/min. 10 mg/mL$_{column}$ corresponds to 1 % loading (expressed as mg$_{target}$/μL$_{column}$), which allows to efficiently perform purification of the desired product in overloaded condition with satisfactory productivity. Since the maximum concentration of DMC is roughly 10 % (v/v) in pure water [22], the "organic" mobile phase (MP-B) used for the gradient elution was a mixture of 20 mM triethylamine phosphate (TEAP) buffer (pH=8 adjusted with H_3PO_4):DMC 90:10%(v/v). Then, the "aqueous" mobile phase (MP-A) was prepared by mixing 90 % of 20 mM TEAP and 10 % of MP-B. The mixed phases were prepared by adding first DMC and then the TEAP buffer. The solution was shaken and sonicated with ultrasound for 30 min until complete dissolution of the bubbles.

The mobile phases prepared for the purification method using EtOH and IPA were the following. MP-A was a mixture of 20 mM TEAP:EtOH (or IPA) 90:10 % (v/v) while MP-B was 20 mM TEAP:EtOH (or IPA) 50:50% (v/v). The details of the purification methods with DMC, EtOH, and IPA are shown in Table 1. During the elution step, the flow rate was 1 mL/min, and fractions were collected every 1 mL. The purification methods were compared with an existing method in ACN already applied for the purification of Icatibant in [28], where the mobile phases were 20 mM TEAP:ACN 90:10% (v/v) as MP-A and a mixture of 20 mM TEAP:ACN 50:50% (v/v) as MP-B. The flow rate during the elution with the latter method was 1.5 mL/min and fractions were collected every 1 mL. A lower flow rate was used when DMC, EtOH and IPA were used as organic modifiers due to the higher backpressure generated by these solvents with respect to ACN.

Table 1
Experimental details used for the purification of Icatibant with the four different solvents.

Modifier	Duration of the gradient (CV)	Flow rate (mL/min)	Variation of organic modifier along the gradient (%)
DMC	10.3	1.0	7.3–10.0
ACN	18.0	1.5	14.8–24.8
IPA	10.3	1.0	14.0–22.0
EtOH	10.3	1.0	20.0–28.0

3.3. Analytical method

For the offline analysis of all collected fractions, an Agilent 1290 Infinity UHPLC system (Agilent, Santa Clara, CA, USA), equipped with a binary solvent pump, a column thermostat set at 50 °C, a diode array detector (DAD) set at 226 nm and an autosampler, was employed. The column was a 250 × 4.6 mm Kromasil 5-100-C18, with particle size of 5 μm and a pore size of 100 Å. The analytical method has already been described in [28]. Diluted standard solutions of Icatibant, used for the calibration curve, were prepared by dissolving the pure peptide, provided by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy), as described for the crude mixture, in a concentration range from 0.1 to 3 g/L. The feed purity measured with HPLC was about 76 %.

4. Results and discussion

This study began with the investigation of the retention behavior of Icatibant by changing the composition of the MP. The MP was a solution of TEAP buffer (pH = 8) modified with variable percentages of four different organic modifiers, namely ACN, DMC, EtOH and IPA. The retention curves of Icatibant are presented as log$k$ vs. φ plots in Fig. 1, being k the retention factor, defined as $k = (V_R - V_0)/V_0$, with V_R the retention volume and V_0 the hold-up volume of the column.

The trend reflects the traditional reversed phase behavior, with a quasi-linear decrease in the logarithm of retention when increasing the organic solvent percentage in the mobile phase. These curves display quite different operative ranges depending on the type of organic modifier that is employed. The most important difference is that, as already observed for small molecules in the first part of this work, with DMC the MP elution power is strongly enhanced. Indeed, it is possible to observe that to achieve a retention factor of $k = 1$ for Icatibant, about 9 %(v/v) DMC in the MP is needed, while higher percentages of the other solvents are required, such as 22 %(v/v) of IPA, 30 %(v/v) of ACN and 35 %(v/v) of EtOH, which shows the lowest elution power. From Fig. 1, it can be also observed that the slopes of the retention curves obtained with ACN, IPA and EtOH are very similar whereas that of DMC is a bit steeper, which implies that a small change on the concentration of organic modifier may have a significant effect on retention, at least for this analyte and in these conditions. However, albeit this may affect reproducibility with respect to the other solvents, no considerable deviations in terms of retention times were observed during the experiments performed in this work. Furthermore, the potential variability associated to the retention curve slope can be avoided through the use pre-mixed mobile phases containing both the aqueous buffer and the organic modifier in different proportions.

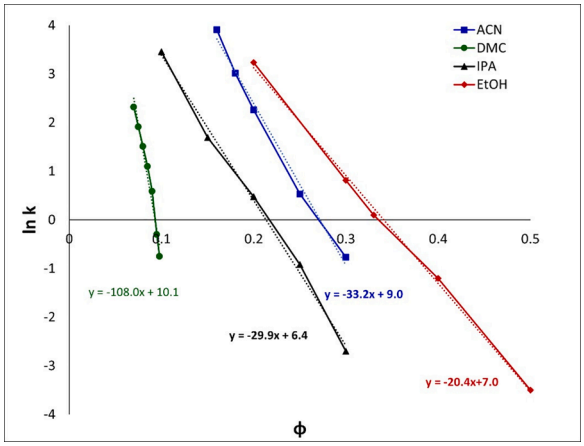

Fig. 1. Dependence of the retention factor, expressed as ln k, on the fraction of organic modifier ϕ in MP, using ACN (blue squares), DMC (green circles), EtOH (red diamonds) and IPA (black triangles).

From the data obtained in the retention study, several overloaded LC methods were developed for the purification of Icatibant by choosing suitable ranges of φ, depending on the nature of the organic modifier (see Table 1 and Fig. 2A–D). As it can be seen, the variation of organic modifier is limited for DMC, due to its higher elution strength. This translates into a smaller consumption (and waste) of organic solvent, with considerable advantages from the disposal and environmental viewpoints.

Focusing on the target peak, it can be noted that its shape is similar for the four solvents, with a diffused front and a shock in the rear part, more or less pronounced, suggesting an anti-Langmuirian adsorption mechanism. However, it can be also observed that the peaks obtained with ACN and DMC show "better" peak shapes if compared to alcohols. This aspect should be carefully taken into account when dealing with complex mixtures, as for peptides, since broader peaks may result in significant peak overlap between the target and impurities, with detrimental effects on the final trade-off between product purity and recovery.

The fractions subtended to the target peaks were collected and off-line analyzed through UHPLC. From the data obtained, it was possible to plot the so-called Pareto curve which shows how the purity varies with the recovery, as shown in Fig. 3. This curve is obtained by first considering the purest fraction and its recovery (upper point on the left). Then, this sample is pooled with the purest adjacent fraction to increase the recovery, and so on until the entire target peak is collected, as described in [28]. By enlarging the collection window, recovery will increase while purity will decrease. The result is a purity-yield trade-off, according to which it is very difficult to obtain high purity and high recovery at the same time.

These curves have been constructed for each of the four solvents used in this work and they provide fundamental information about the performance of a purification method on preparative scale. For the sake of clarity, data related to recovery, purity and productivity are reported in Table 2. From Fig. 3 and Table 2 it can be noted that DMC provides purification performance comparable to ACN, since their curves are almost superimposed. Concerning the purest fraction, i.e., the first point on the left, either ACN or DMC lead to a purity of about 100 %, while it is slightly lower with EtOH and IPA (roughly 99 %).

If the other limiting case is considered, corresponding to the maximum yield (achievable when the entire product collection window is pooled), it can be observed that with all solvents roughly 100 % recovery has been obtained. In this case, ACN provides the highest purity (98 %), followed by DMC (97 %) and alcohols (96 %). All solvents show a comparable gain in purity, roughly 30 %, with respect to the initial feed, whose purity was 76 %. It is worth noting that the productivity measured at 100 % recovery is the same for all solvents except for ACN, for which it is lower due to the longer gradient duration (see Eq. (3)).

As it was pointed out, the two end points of the Pareto curve are useful to check the goodness of different purification methods, in terms of yield-purity trade-off. Furthermore, the pharmaceutical contest demands high purity values for the isolated final product, usually >98 %. Accordingly, a peptide purity of 98.5% has been set as target for the experiments performed in our study. In Table 2, recovery and productivity values have been calculated in correspondence with this purity for all the solvents. As it can be noted, both ACN and DMC lead to the highest recovery (roughly 94 %) if compared to EtOH (65 %) and IPA (80 %). This result indicates that the former solvents are able to provide highly pure product with a minimal loss of mass (around 6 %). Concerning productivity, the maximum value has been obtained with DMC. Also in this case ACN shows a productivity value smaller than DMC, due to the longer gradient used.

All the potential alternative solvents to ACN have shown interesting outcomes for the purification of Icatibant. Nevertheless, it has been seen that, for this specific case, especially DMC can provide very similar results to ACN, in terms of recovery and purity. It is worth mentioning that depending on the imposed target purity also EtOH or IPA can be

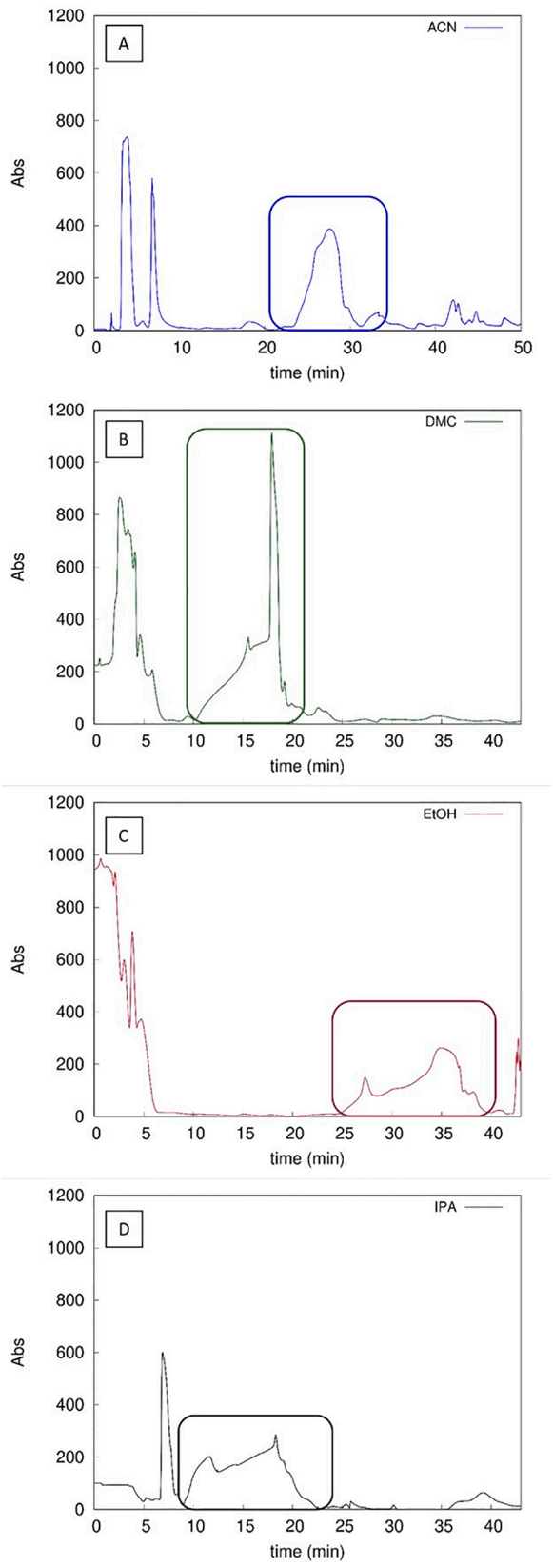

Fig. 2. UV profiles of Icatibant along the gradient step obtained using ACN (A), DMC (B), EtOH (C) and IPA (D). The target peak has been highlighted with a box. Time=0 identifies the beginning of the gradient.

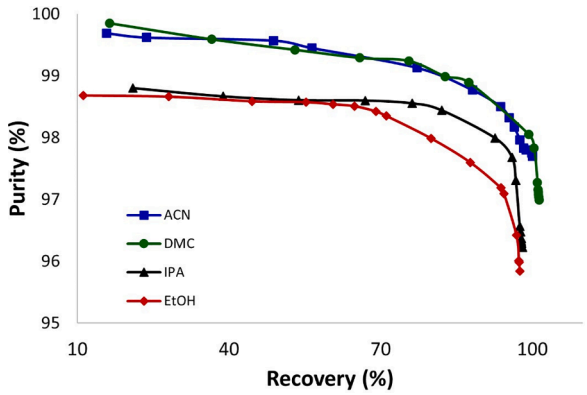

Fig. 3. Pareto curves for the purification of Icatibant by using ACN (blue squares), DMC (green circles), EtOH (red diamonds) and IPA (black triangles).

Table 2
Comparison of the performance parameters using different organic modifiers, at the two extremities of the Pareto curve (highest purity and highest yield) and at a target purity equal to 98.5%.

ORGANIC MODIFIER	RECOVERY (%)	PURITY (%)	PRODUCTIVITY (mg/L/h)
ACN	**15.8**	**99.7**	**2.8**
	99.9	**97.7**	**11.9**
	94.0	**98.5**	**11.2**
DMC	**16.4**	**100.0**	**2.4**
	99.9	**96.9**	**14.1**
	93.5	**98.5**	**13.9**
EtOH	**11.2**	**98.7**	**1.6**
	97.4	**96.0**	**13.9**
	64.8	**98.5**	**9.4**
IPA	**21.0**	**98.8**	**2.3**
	98.1	**95.8**	**14.2**
	79.0	**98.5**	**11.2**

effectively used as green alternatives to ACN.

5. Conclusions

The replacement of ACN with greener solvents is becoming an urgent need in the biopharmaceutical industry. In this study, several potential alternative solvents, namely DMC, EtOH and IPA, have been tested for the purification of a therapeutic peptide, Icatibant.

This study revealed that all the solvents selected are able to effectively purify Icatibant, with roughly 100 % recovery and > 95 % purity. Nevertheless, when a pharmaceutically acceptable purity is selected (e.g. > 98.5 %), ACN and DMC show much better results in terms of recovery compared to alcohols. Remarkably, the highest productivity is obtained when using DMC as organic solvent.

In this context, DMC can be considered as a promising candidate to be used also in preparative conditions for the isolation of biopharmaceuticals. In particular, as demonstrated also in the companion article for some small molecules, this solvent shows a higher elution strength with respect to ACN, as well as the other alcohols that have been tested (in particular EtOH, which is among the solvents available today in terms of greenness). These features will potentially translate into shorter runs, less instrumentation usage, and a considerable decrease of organic solvent waste, the disposal of which is generally an unnecessary cost and burden to the environment. All these aspects are very important from the point of view of the greenness of the process. The overall aim of these two companion articles is to propose DMC as a green alternative to the well-established ACN, IPA and EtOH, an alternative which had almost never been investigated before for chromatographic applications, especially in overloaded conditions. Its suitability for the purification of a peptide mixture of industrial interest was demonstrated at semi-preparative level, showing comparable performance result respect to ACN. Similar results have to be expected at industrial preparative level, ensuring to keep the same stationary/mobile phases and the% of product loaded respect to the dimension of the column selected.

The benefits obtained with DMC could even be amplified in the future, by employing it in highly productive manufacturing processes possibly based on continuous multicolumn chromatographic techniques. Multicolumn Countercurrent Solvent Gradient Purification (MCSGP), for instance, has led to impressive improvements in productivity and solvent consumption in the purification of Icatibant through ACN, with respect to traditional single-column chromatography [28]; therefore, even better results are expected by employing DMC with this technique, contributing to develop greener manufacturing processes of peptides [31].

CRediT authorship contribution statement

Desiree Bozza: Investigation, Validation, Writing – original draft, Visualization. **Chiara De Luca:** Methodology, Formal analysis, Writing – original draft. **Simona Felletti:** Methodology, Formal analysis, Writing – review & editing. **Matteo Spedicato:** Investigation, Validation. **Francesco Presini:** Validation, Data curation. **Pier Paolo Giovannini:** Validation, Data curation. **Marco Carraro:** Methodology, Data curation, Resources. **Marco Macis:** Methodology, Data curation, Resources. **Alberto Cavazzini:** Supervision, Funding acquisition, Writing – review & editing. **Martina Catani:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Antonio Ricci:** Conceptualization, Resources, Supervision, Project administration, Writing – review & editing. **Walter Cabri:** Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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