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FULL PAPER

Future medicinal chemists experience flow chemistry: optimization by experimental design of the limiting synthetic step to the antifungal drug econazole nitrate

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Abstract
A practical experience to showcase the potential of flow technology in the synthesis optimization of drugs has been developed and carried out by fourth year undergraduate students in Pharmaceutical Chemistry and Technology (CTF) at the University of Perugia. In particular, we present an experiment aimed at optimizing the monobromination reaction of 2′,4′-dichloroacetophenone, the limiting step to the synthesis of the antifungal drug econazole nitrate. Throughout the experiment, the students learn how to integrate flow chemistry and experimental design to expedite experimental screening and reaction optimization. Moreover, the students have the possibility to exploit the use of automation to improve data generation and reduce human intervention in repetitive, expensive or hazardous experiments.

Keywords Continuous flow chemistry · Experimental design · Monobromination reaction · Synthesis optimization · Undergraduate laboratory practice

Introduction

In the last years, flow chemistry has emerged as a key enabling technology that can complement or replace batch chemistry to meet modern synthesis criteria, such as efficiency, sustainability, and safety [1]. In drug discovery, continuous flow technology is increasingly exploited to rapidly synthesize compound libraries for hit-to-lead explorations and to streamline chemical processes of active pharmaceutical ingredients (APIs) and drug candidates in clinical development [2–4]. Indeed, flow chemistry offers several advantages including the accurate control over the reaction parameters that translates into a higher product quality and robust synthesis, the efficient mixing and heat/mass transfer that improve reaction rates and productivity, as well as the wider exploration of the chemical space [5]. Furthermore, the integration of flow synthesizers with downstream operations, automation and in-line monitoring reduces manual operations ensuring the rapid experimental screenings and compounds synthesis [6, 7].

Despite the growing applications of continuous flow chemistry in both academia and pharmaceutical companies, this technology is not often taught within the education programmes of undergraduate students in chemistry and related disciplines. Examples of implementations in training courses are few and limited to institutions hosting researchers active in the field. In 2015 we have introduced flow chemistry in the teaching course ‘Laboratory of extraction and synthesis of drugs’ as we thought students of the master degree in Pharmaceutical Chemistry and Technology (Chimica e Tecnologia Farmaceutiche, CTF) could benefit of a broader education with regard to chemical technologies. The major objective of the course is to provide the theoretical basis and practical aspects in carrying out the synthesis and purification of APIs using common laboratory equipment as round-bottom flasks.

The last synthesis that students realize in the laboratory practical classes consists in the multistep preparation of econazole nitrate (**1**) (Scheme 1) [8], an antifungal drug that is administered topically for the treatment of dermatomyeoses and vaginal candidomim [9]. The synthesis is conducted under standard batch conditions and starts with the bromination reaction of 2′,4′-dichloroacetophenone (**2**) using *N*-bromosuccinimide (NBS) (1 equiv.) and *p*-toluensulfonic acid

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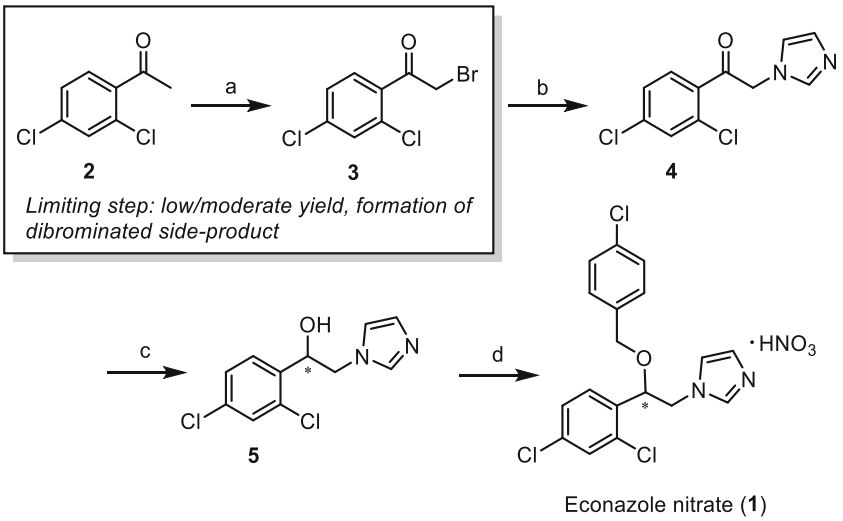


Scheme 1 Synthesis of econazole nitrate (**1**) from 2′,4′-dichloroacetophenone (**2**) [8]. *Reagents and conditions: a)* NBS (1 equiv.), *p*TSA·H_2O (0.1 equiv), 80 °C, 1 h; *b)* imidazole (3 equiv.), $CHCl_3$, reflux, 2 h; *c)* $NaBH_4$ (2 equiv.), MeOH, r.t., 1 h; *d)* NaH (3 equiv.), *p*-chlorobenzylchloride (1.5 equiv.), DMF, 0 °C > r.t., 1 h; then HNO_3, Et_2O, 0 °C.

(*p*TSA) (0.1 equiv.) in neat conditions at 80 °C. The crude reaction mixture is then treated with imidazole (3 equiv.) in refluxing $CHCl_3$ to give the imidazole intermediate **4** after silica gel chromatography.

The pure imidazole derivative **4** is reacted with $NaBH_4$ in MeOH at 25 °C to furnish a racemic mixture of the alcohol **5** that, after treatment with NaH (0 °C) and *p*-chlorobenzyl chloride in DMF at 25 °C, affords the target product **1**. Finally, the addition of HNO_3 to an ethereal solution of **1** allowed students to obtain the precipitation of a pure white solid. The overall yield of the synthetic route is generally low or moderate and ranges from 25 to 40% [8] because of poor reproducibility and efficiency of the bromination step. Indeed, the reaction is difficult to control due to its fast reaction rate and exothermic character that lead to the formation of the dibrominated side-product.

In this context, it would be appealing from a didactic point of view to teach students how to use flow technology to solve synthesis limitations and, in particular, to improve the first limiting step to the synthesis of econazole (**1**). Thus, we have developed an experiment aimed at optimizing the selective α-bromination of **2** by an integrated approach based on flow chemistry and statistical design of experiments (DoE) [10–12]. The experience includes (a) the study of the reaction under investigation to acquire the theoretical background, (b) the batch screen to select the model reaction, (c) the execution and analysis of the DoE experiments using flow devices, and (d) the identification of optimal conditions for gram scale synthesis. As a work extension, we also illustrate the conduction of flow experiments using automation.

Before experiencing

Before practicing, students learn about the concepts, devices and applications of continuous flow chemistry. Moreover, the laboratory exercise is preceded by the study of the reaction to provide students with the appropriate background to perform the experiments and interpret the results. Further teaching on statistical-based methods is also important to have the basic theory of experimental design. Students are also made aware about safety hazard indications for all the chemicals employed in the experiments (see Chemicals, Safety Data and Hazards, Supporting Information).

Analysis of the reaction

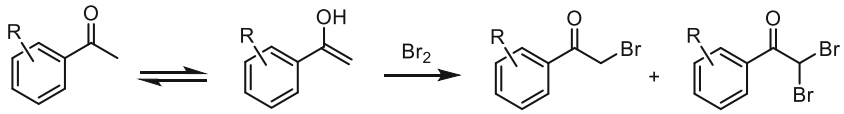
The α-monobromination of acetophenones can be carried out using diverse conditions and brominating agents [13]. Brønsted or Lewis acids can be employed as catalysts, with or without coordinating solvents and in neat conditions. The reaction is exothermic with a fast reaction rate and it is difficult to control under 'traditional' batch conditions. The brominated ketone can easily react to generate the dibrominated product (Scheme 2). In 2012, the α-bromination of acetophenone was translated from batch to flow conditions using 1.5 equiv. of Br_2 in the presence of HBr in 1,4-dioxane at 20 °C by means of microreactor technology [14]. After optimization, an excellent selectivity of monobromination was achieved on a preparative gram scale.

In our case, for safety reasons, we decided to conduct the experiment using freshly crystallized NBS. Indeed, although widely used, Br_2 evaporates easily in a red vapor that is very toxic by inhalation. During class, students are invited to discuss the reaction mechanism as well as the effects of experimental parameters and substrate substituents on the reaction outcome.

Batch screen

The first part of the study relies on the conduction of a series of batch experiments to identify the appropriate model reaction,

Scheme 2 Bromination reaction of acetophenones.



the relevant experimental parameters, and relative range of investigation for the DoE optimization under flow conditions. In particular, students investigate the effect of solvent (CH_2Cl_2, 1,4-dioxane, CH_3CN), NBS equiv. (1.1 and 2), the catalyst (*p*TSA, HBr, H_2SO_4), and the temperature (25 and 60 °C) (Table S1, Supporting information). Catalysts are selected because of their availability and low costs [15].

Reactions are performed by a group of 20 students using 1 mmol of starting material **2** and the minimum amount of solvent that guarantees the solubilisation of all the reaction components. After work-up, the crude reaction mixtures are analyzed by NMR spectroscopy for the determination of the reaction yield (for a detailed procedure see the Supporting Information). The collected data (reaction yields as determined by NMR) are shared so that the students can analyze the results and propose their own model reaction. Under the investigated conditions, 1,4-dioxane was found as the best solvent, while a slight excess of NBS was sufficient to afford the desired product **3** unless the reaction was conducted in CH_3CN. The best performance was obtained using 1.1 equiv. of NBS and 0.1 equiv. of HBr in 1,4-dioxane at 60 °C for 1 h (Table S1, Supporting information). These conditions were therefore selected for next optimization stage.

The entire batch screen requires maximum four hours *per* pair of students, including carrying out the reaction and the ^{1}H-NMR analysis. The results of the screening experiments are collected, shared via e-mail by the teacher, and discussed in class before starting the flow experiments.

Reaction optimization by experimental design and flow technology

Having defined the model reaction, students participate to set-up an experimental design for three factors suspected to mainly influence the reaction: temperature (T) (40–80 °C), residence time (τ) (30–80 min) and NBS stoichiometry (1.1–2.0 equiv.) (Table 1). The experimental matrix is based on a central composite design (CCD) composed by fifteen experiments plus five replicates at the central point (Table 2) [16].

Students then start the actual experimentation in which they vary the flow rate and the temperature, collect the run, perform the work-up, and proceed to prepare a sample of the reaction crude for the NMR analysis. Reactions are performed using commercial, widely available equipment that comprises two pumps, a T-piece, a 10 mL coil reactor, and a back pressure regulator. Experiments are conducted by pumping two stock solutions: the first one contains 2′,4′-dichloroacetophenone (**2**) (0.5 mmol, 1 M) and HBr (0.1 equiv.) in 1,4-dioxane, and the second is a solution of NBS (0.5 M) in 1,4-dioxane (Fig. 1). Reagents solutions are mixed in a T-piece and flowed into a 10 mL reaction coil heated at the selected temperature. The output was diluted with EtOAc and quenched with 5% aqueous $Na_2S_2O_3$ before collection. After collecting the product, students can clean the flow set-up with 1,4-dioxane to remove residuals that are still in the outlet channel and

Table 1 Variable settings for reaction optimization

Variable	Unit	Range
Temperature (A)	°C	40–80
Residence time (B)	min	30–80
NBS stoichiometry (C)	equiv.	1.1–2.0

Table 2 CCD: experimental matrix and measured responses

Entry	Factor A Temperature (°C)	Factor B τ (min)	Factor C Equiv. NBS	2 (%)[a]	3 (%)[a]	6 (%)[a]
1	72	70	1.82	0	63	37
2	60	55	1.55	6	80	14
3	48	40	1.28	30	68	2
4	60	55	1.55	4	82	14
5	72	70	1.28	4	79	17
6	48	40	1.82	15	70	15
7	72	40	1.28	20	76	4
8	48	70	1.82	2	74	24
9	72	40	1.82	11	70	19
10	60	80	1.55	0	71	29
11	48	70	1.28	11	82	7
12	60	55	1.55	5	82	13
13	60	55	2.00	7	68	25
14	40	55	1.55	8	82	10
15	60	55	1.10	22	76	2
16	60	30	1.55	25	65	10
17	60	55	1.55	5	81	14
18	60	55	1.55	4	81	15
19	60	55	1.55	5	80	15
20	80	55	1.55	4	74	22

[a] Yields were determined by calibrated ^{1}H-NMR analysis of the crude mixtures after quenching with 5% (w/v) aqueous solution $Na_2S_2O_3$ [17]

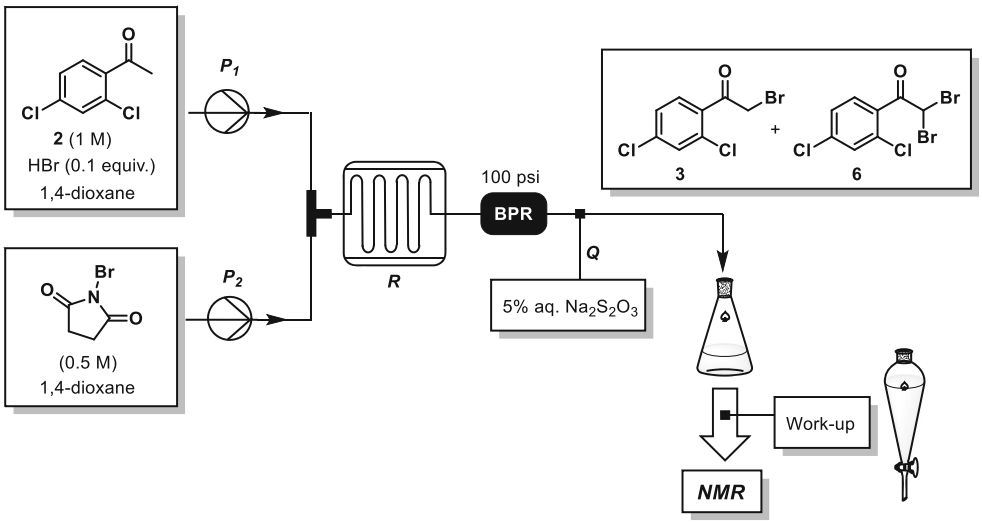


Fig. 1 Flow set-up used for DoE optimization of the bromination of 2′,4′-dichloroacetophenone (**2**). BPR: back pressure regulator; P_{1-2}: pumps; R: 10 mL reactor coil; Q: quenching

prepare the system for the next factor settings. After work-up, the organic layer is washed with aqueous $NaHCO_3$, dried with sodium sulfate and concentrated using a rotavapor before NMR analysis. Next, students report their results as the percentage of desired product **3**, dibrominated byproduct **6**, and unreacted starting material **2** (Fig. 1).

Each experimental run can be carried out by the students in about three hours, working in pairs. The laboratory is provided with three flow devices allowing to complete the entire optimization experiments within three days.

On the fourth day, the data acquired are fitted into quadratic equations (Eq. 1–3) furnishing three mathematical models and the corresponding response-surface plots as illustrated in Fig. 2.

$$\%(\mathbf{2}) = 4.83 - 2.17A - 7.36B - 4.53C + 1.35AC + 1.35BC + 2.74B^2 + 3.45C^2 \quad (1)$$

$$\%(\mathbf{3}) = 80.97 - 1.41A + 1.75B - 3.02C - 2.70AB - 1.96AC - 2.46BC - 0.90A^2 - 4.44B^2 - 3.02C^2 \quad (2)$$

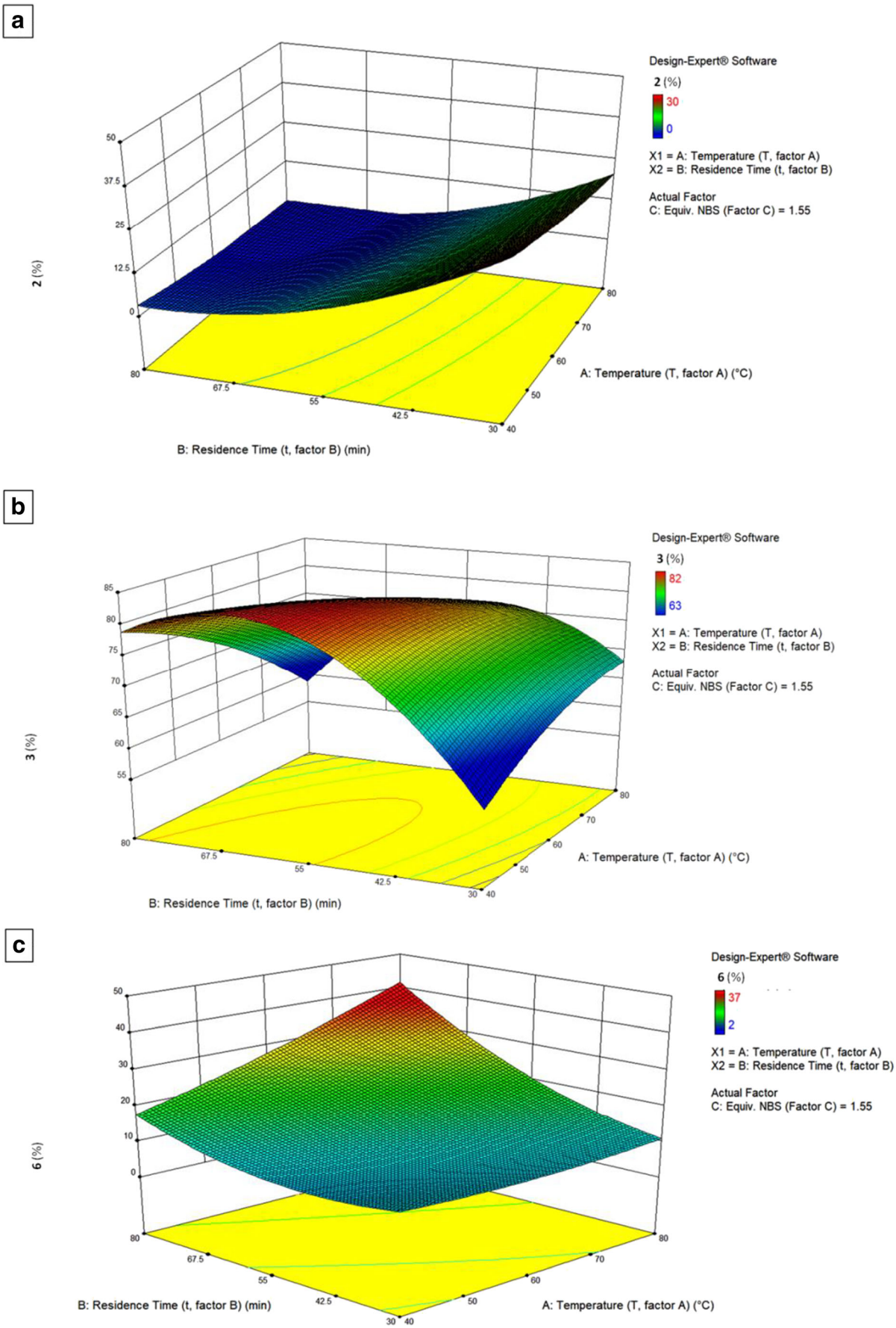
$$\%(\mathbf{6}) = 14.20 + 3.58A + 5.60B + 7.55C + 2.09AB + 1.10BC + 1.70B^2 \quad (3)$$

At this point, students can analyze statistical parameters (ANOVA analysis) to assess significance and predictivity of the method (Tables S2-S4, Figs. S1-S3, Supporting Information). Moreover, to identify the optimal factors they can feed the DoE software with specific optimization criteria. As an example, the maximization of the desired product **3** and minimization of the side-product **6** furnished different solutions that can be ranked in desirability order [18]. Among these, the conditions reported in Table 3 are adopted to prove the reaction on gram scale [19]. As a results, the method enables the synthesis of **3** in 79% yield along with 4% of the dibromominated analog **6** (Fig. S5, Supporting Information), showing a high degree of correlation between the predicted and experimental results (Table 3).

A taste of automation

In an extension of the experiment, we also show the use of automation with flow synthesizers to execute the chemical experiments with reduced manual interventions. Such an approach can significantly accelerate process optimization and compounds synthesis especially when integrated with design software, predictive statistical tools, in-line analysis, and downstream operations [2]. In our case, reactions are run by means of an automated reagent injector equipped with two channels dedicated to the 2′,4′-dichloroacetophenone (**2**) and the NBS stock solutions, removable vial racks and two internal loops. An UV detector can be inserted to monitor the reaction and the progress of the experiment. The automated injector is connected to a laptop and controlled by a software that allows the student to draw the desired flow set-up (Fig. S6, Supporting information), build the experiments table, and monitor the progress of the experiment in real time [20]. The injector fills the two reagents channels, loads the reagents into the loops and switches the valves so that

Fig. 2 Tridimensional response surface plot related to 2′,4′-dichloroacetophenone **2** (a), monobrominated product **3** (**b**) and dibrominated product **6** (**c**) using 1.55 equiv. of NBS ▶


a
Design-Expert® Software
2 (%)
30
0
X1 = A: Temperature (T, factor A)
X2 = B: Residence Time (t, factor B)
Actual Factor
C: Equiv. NBS (Factor C) = 1.55
2 (%)
50
37.5
25
12.5
0
80
67.5
55
42.5
30
B: Residence Time (t, factor B) (min)
40
50
60
70
80
A: Temperature (T, factor A) (°C)
b
Design-Expert® Software
3 (%)
82
63
X1 = A: Temperature (T, factor A)
X2 = B: Residence Time (t, factor B)
Actual Factor
C: Equiv. NBS (Factor C) = 1.55
3 (%)
85
80
75
70
65
60
55
80
67.5
55
42.5
30
B: Residence Time (t, factor B) (min)
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A: Temperature (T, factor A) (°C)
c
Design-Expert® Software
6 (%)
37
2
X1 = A: Temperature (T, factor A)
X2 = B: Residence Time (t, factor B)
Actual Factor
C: Equiv. NBS (Factor C) = 1.55
6 (%)
50
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10
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80
67.5
55
42.5
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B: Residence Time (t, factor B) (min)
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80
A: Temperature (T, factor A) (°C)

Table 3 Predicted and experimental results for the selected optimisation criteria

Optimization criteria[a]	Conditions	Yield (%)[b]					
		2		**3**		**6**	
		P	E	P	E	P	E
• Maximise **3** • Minimise **6** • **2**: none • A, B and C in range	A = 48 °C B = 64 min C = 1.23 equiv. NBS	15	17	81	79	4	4

[a] Reaction performed using the flow set-up depicted in Figure 1. [b] Determined by calibrated ^{1}H-NMR analysis of the crude mixture after quenching with 5% (w/v) aqueous solution $Na_2S_2O_3$ [17]. P: predicted; E: Experimental

the reactants can enter into the reactor. At the same time, the software regulates the reactor temperature and the pump flow rates according to the planned experimental conditions. While the experiment is running, both the needles and the loops are washed with 1,4-dioxane before running the next experiment.

Assessment and possible experiment extension

The students are assessed based on their practical performance, skills and the written report. The practical performance and skills of the students are evaluated considering their ability to implement the experimental procedures, to realize the flow set-up, and to execute the flow experiment. After the laboratory practice, students have to write a report describing the procedures used, eventual variations with respect to the standard protocol, all the relevant observations noted down during the experimentation, and the results obtained. The report should be personal, complete and accurate, furnishing adequate conclusions about the meaning of their observations, and suggestions for potential implementations. Over the years, the experiment has been well received by the students with positive feedbacks and good quality reports.

This flow experiment is the last one in the training course. Hence, in principle, the students can conduct the entire experience including the batch reactions, the flow set-up and execution, the practical demonstration on the use of automation, and the discussion of the results between the study stages, in five working days. However, there is the possibility to extent or adapt the experiments according to the teachers and students need, laboratory space and equipment. Some options are reported below:

a) Batch screening experiments can include the evaluation of other reagents, solvents, experimental parameters and relative ranges, and reaction conditions. The DoE matrix can be then adjusted accordingly.
b) The reaction yield are determined by calibrated ^{1}H-NMR analysis. Other analytical techniques such as HPLC, GC-MS, or LC-MS could be also used, eventually in-line. Calibration curve can be setted-up for a more accurate determination of the reaction yield and composition.
c) Students can investigate different flow set-up, the use of diverse mixing elements, the integration of membrane separators and in-line analysis.
d) Other experimental designs, such as D-optimal, Plackett-Burman, and fractorial design can also be applied. Furthermore, students can choose alternative optimization criteria.

Conclusions

A laboratory experiment that shows the potential of flow chemistry in the synthesis optimization of pharmaceutical compounds was developed for undergraduate students of the master's degree course in Pharmaceutical Chemistry and Technology (CTF) at the University of Perugia. The scope of the degree course is to train professionals and researchers in key disciplines of drug discovery, including medicinal chemistry. It is therefore our belief that future synthetic medicinal chemists should be educated in the use of enabling chemical technologies to tackle the future challenges for a more sustainable chemistry and drug discovery. While we will continue teaching 'conventional' approaches, we should devote attention to the tremendous opportunity of adopting technological solutions as continuous flow chemistry. Provide undergraduate chemists with the concepts, potential and practice of flow chemistry would certainly straighten their ability to realize more efficient and greener chemical processes.

Herein, we have described an integrate experiment designed to solve limitations of the first step in the synthesis of the drug econazole nitrate (**1**) by DoE-assisted continuous flow optimization. The presented experiment was useful to rapidly define a new method for the efficient and selective α-bromination of 2′,4′-dichloroacetophenone (**2**) to increase the overall yield of econazole nitrate (**1**) and facilitate purifications between steps. The experiment needs cheap and readily available reagents and the experiments are achievable using easy-to-made coil reactors [21], pumps, and common laboratory glassware. We believe this experiment can be useful to get chemistry students acquainted with the basis, tools and use of flow chemistry.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41981-020-00136-z.

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8. The synthesis is realized in five working days. The overall yield is calculated as average of the results obtained by around 350 undergraduate students since 2013
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16. Design of experiments and statistical data analysis was performed using Design-Expert® v. 9 (Stat-Ease Inc., Minneapolis, MN – USA)
17. The reaction yield was assessed by calibrated ^{1}H-NMR analysis of the crude reaction mixture after quenching with 5% (w/v) aqueous solution $Na_2S_2O_3$. In particular, the percentage of unreacted **2**, monobrominated **3** and dibrominated **6** was assessed considering the ratio between the integrals related to the hydrogen nuclei at the α position to the ketone group (singlet at 2.67, 4.52 and 6.75 ppm, respectively). Dimethyl sulfone (standard for quantitative NMR, TraceCERT®) was used as the internal standard for integrals normalisation (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Brochure/1/qnmr-brochure-rjo.pdf)
18. The adopted optimization criteria were driven by the fact that the crude of the bromination step is directly submitted to the substitution reaction with imidazole. At this regard, the presence of dibrominated side product **6** should be minimized as the corresponding disubstituted analog is difficult to remove by silica gel chromatography
19. The gram scale flow synthesis of **3** is performed by the student tutors and the results are shown to the students in class. The synthesis is performed by pumping a solution of **2** (10 mmol, 1 M) and HBr (0.1 equiv.) in 1,4-dioxane and a solution of NBS (0.5 M) in 1, 4-dioxane at 45 μL min^{-1} and 111 μL min^{-1}, respectively. The solutions are mixed together in a T-shaped mixing element and entered the coil reactor (10 mL) heated at 48 °C (Figure 1). The reactor outcome was diluted with EtOAc and quenched with 5% wt. aqueous solution of $Na_2S_2O_3$
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