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Fast and fully automated analytical method for the screening of residues of aziridine and 2-chloroethylamine in pharmaceutical active principles

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ABSTRACT

A simple, fast and fully automated method for the screening of aziridine (AZD) and 2-chloroethylamine (CEA) in active pharmaceutical ingredients (API) has been developed. The method is based on the in-fiber derivatization of the amines extracted from the sample headspace (previously dissolved or suspended in alkaline water) with 2,3,4,5,6-pentafluorobenzoyl chloride (PFBCl) previously adsorbed in the PDMS/DVB solid phase microextraction (SPME) fiber. The derivatives formed are further desorbed and analyzed in a gas chromatograph with negative ion chemical ionization mass spectrometry (GC–NCI-MS) using methane as reagent gas. The different operational parameters of the procedure have been optimized to get highest sensitivity. The validation of the method, however, revealed a poor repeatability, particularly evident in water-soluble APIs (RSD > 20% for AZD). In spite of that, the low detection limits (1–3 ng g^{-1} for AZD and CEA), speed (44 min total analysis time) and automation make that this method can be satisfactorily used as screening tool to accept or reject API batches attending to their volatile amine content and a critical specified value derived from the 1.5 μg/day Threshold of Toxicological Concern (TTC) and maxima daily dosages. This was shown by analyzing seventy-five fluvoxamine maleate samples containing known levels of AZD and CEA (between 0.05 and 1.05 μg g^{-1}) in intermediate reproducibility conditions to get reliable estimations of precision and linearity. From these data, acceptance, rejection and non-conclusive areas of response are defined for both analytes at different confidence and replication levels using normal statistics. The method was satisfactorily applied to real fluvoxamine maleate samples.

1. Introduction

Some volatile amines are used in the synthesis of active pharmaceutical ingredients (API) and hence may be present in small or trace amounts in the final products. Some other volatile amines are formed upon degradation of API or of some of the reagents used during the synthesis. Many of those volatile amines are dangerous compounds because of their toxicity and because they are also potential precursors for N-nitrosamines which are powerful carcinogenic agents [1–7]. This is for instance the case of 2-chloroethylamine, often used in the synthesis of many API containing amine functional groups. Residual amounts of 2-chloroethylamine can react and cycle to produce aziridine (AZD), a powerful carcinogenic agent [6,8–10]. Because of this, the synthesis and development of drugs requires a strict control of the potential volatile amines remaining in the API, and hence, there is a need for adequate analytical methods for the quantitative determination of these compounds in API samples at μg g^{-1} [11] level. This is the sensitivity level that would be required in most cases to ensure that, in the absence of a well defined threshold of genotoxicity, the 1.5 μg/person/day TTC level is not reached [12].

Because of their volatility and the low levels at which they must be analyzed, the a priori preferred technique for the analytical determination of volatile amines should be gas chromatography [13,14]. However, the direct gas chromatographic analysis of these compounds is quite difficult because of their high reactivity, polarity, hydrogen-bonding character, alkaline character, and high water solubility [1,4]. Although there are some commercial chromatographic columns proposed for the GC-separation of underivatized amines and there are some works reporting on its use [1], their use for trace amine determination is not straightforward. It is because of this that most of the methods used are based on the chemical derivatization of the amines. The derivatization reaction seeks to decrease polarity, improve volatility, reduce chemisorption-related problems and also improve the detectability of the molecules. The derivatization process most often requires multi-step methodologies and is time-consuming. A review about the reactions used for amine analysis has been presented [13]. One of the most widely

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used reagents is 2,3,4,5,6-pentafluorobenzoyl chloride which forms the corresponding pentafluorobenzoyl amides. These compounds are less alkaline, less polar and can be selectively and sensitively detected by electron capture detection or negative ion chemical ionization mass spectrometry (NCI-MS) [3,7,15–17].

However, the formation of amine derivatives in the presence of amine-containing API can turn to be difficult [6,18], may require further separation steps and if these are not very efficient, the final extracts may still cause chromatographic problems derived from the presence of non-volatile API-derivatives. For instance, the method developed by de Haan et al. for the analysis of 2-chloroethylamine (CEA) and aziridine (AZD) is based on their derivatization with acid chlorides in liquid media. Reaction takes place in a two-phase system (aqueous–organic solvent) in which API has to be removed before derivatization. The method includes a procedure for water-soluble APIs and a second one for the water insoluble ones in which a liquid–liquid extraction before GC is required [6]. The procedure is tedious, requires skilled work and the presence of traces of derivatized API in the extract makes the chromatographic inlets to become soon dirty, requiring frequent maintenance. May et al. presented a method for the determination of aziridine in API by HPLC using derivatization and UV-detection. In order to avoid the strong interference caused by the API, the sample had to be distilled [18]. Similarly, Bowman et al. developed an electrophoretic method for the analysis of other volatile amines using a conductivity detector. In this case, the analysis of real samples required the separation of the API in a C18 cartridge [19]. It can then be summarized that most of the proposed procedures are relatively labor-intensive, difficult to automate and that some of them are prone to suffer problems related to the presence of non-volatile derivatized API in the extract. The most obvious way to impede this last problem is carrying out the derivatization reaction in the sample headspace, since the volatility of most APIs is negligible in comparison with that of AZD and CEA. This is the strategy explored in this paper, whose main aim is to develop a fast, simple and fully automated analytical method for the screening of trace amounts of AZD and CEA in APIs. The selected strategy for carrying out the derivatization is based on the use of a SPME fiber previously exposed to the vapors of derivatization reagent using a robotic autosampler.

2. Materials and methods

2.1. Materials

2,2,2-Trifluoroethylamine (TFEA) >98% from Fluka (Steinheim, Germany), aziridine stabilized (AZD) from Menadiona (Barcelona, Spain), fluvoxamine maleate (FVX), 2-chloroethylamine hydrochloride (CEA), 1-ethylpropylamine (EPA) 97% and 2,3,4,5,6-pentafluorobenzoyl chloride (PFBCl) 99% were obtained from Aldrich (Steinheim, Germany), sodium hydroxide, sodium carbonate and toluene were from Panreac (Barcelona, Spain). Glycine (GLY) and L-phenylalanine (PHE) reagent grade were obtained from Sigma (Steinheim, Germany). Purified water was obtained in a Milli-Q system from Millipore (Billerica, USA). Polidimethylsiloxane/divinylbenzene SPME fibers (PDMS/DVB), 65 μm, were from Supelco España (Madrid, Spain).

2.2. Gas chromatography–mass spectrometry

The gas chromatograph–mass spectrometric system (fast quadrupole type) was a QP-2010 from Shimadzu (Duisburg, Germany). The chromatographic column was a SPB-1 Sulfur 30 m × 0.32 mm i.d., 4 μm film thickness from Supelco España (Madrid, Spain). Initial temperature was 50 °C, kept for 4 min, then raised at 25 °C min^{-1} to 200 °C and finally to 280 °C at 15 °C min^{-1}, kept for 5 min. Desorption of analytes from the fiber was directly carried out in a split/splitless injector with a SPME liner kept at 250 °C. Injection was in splitless mode (splitless time 3.5 min). After that, split ratio was set at 1:10. Carrier gas was He at a constant linear velocity of 50 cm s^{-1}.

Mass spectrometric detection was in NCI mode using methane at 3 bar as reagent gas. The ion source temperature was 220 °C while interphase was kept at 250 °C. Detection was in SIM (Single Ion Monitoring) mode, taking the ions with m/z 209, 237 and 273 from 10.5 to 12.5 min at 0.20 points s^{-1} and finally from 13.21 to 16 min the ions with m/z 55, 217 and 226 at 0.20 points s^{-1} were monitored. The ions used in the analysis were 209 for PFB-AZD, 55 for PFB-CEA, 273 for PFB-TFEA and 226 for PFB-EPA. The other ions were used as qualifiers (237 for PFB-AZD and 217 for PFB-CEA) to confirm without doubt the identity of the peak.

2.3. Study of reagent sorption kinetics

In order to study the kinetics of the sorption of reagent in the fiber, the headspaces of reagent stock solutions (from 0.1 to 1.4%, w/v) in toluene were extracted with the PDMS/DVB fiber at different temperatures (30, 40 and 60 °C) and times (5, 10, 20 and 40 min). The amount of reagent taken in the fiber was determined by desorbing it for 2 min in a split/splitless injector at 250 °C with a split ratio 1:600. The column was a Factor Four VF-5ms 20 m × 0.15 mm i.d., 0.15 μm film thickness from Varian (Walnut Creek, CA, USA). The oven temperature was 200 °C for 2 min, and then raised to 280 °C at 50 °C min^{-1}, kept for 10 min. Carrier was He at a constant flow of 0.91 ml min^{-1}. Detection was in NCI mode. The study of the deactivation of the reagent was similarly carried out by exposing the fiber containing the reagent to the headspace of alkaline aqueous solution. The elimination of the excess of reagent was also similarly studied. In this case, the reagent remaining after the standard reaction (see proposed procedure) was measured and the effect of submerging the fiber in aqueous alkaline solutions on the concentration of remaining reagent was also determined.

2.4. Proposed procedure

Weigh 100 mg of API in a standard 20 ml headspace SPME vial, suspend or dissolve in 5 ml of pure water and add 100 μl of NaOH 10 M. Add 10 μl of aqueous solution containing the internal standards TFEA and EPA at 10 mg l^{-1} and put the vial in the autosampler tray. The API should have been either perfectly dissolved (hydrophilic API) or should have formed an emulsion (hydrophobic API), but no solid crystals should be observed. It should be noted that for insoluble crystalline samples that do not form emulsions, any analyte trapped in the solid matrix will not be detected.

The automated process takes place in a combi-Pal autosampler from CTC analytics (Zwingen, Switzerland) equipped with a 6-port incubation unit, a sample tray and a SPME fiber conditioning unit. All the instructions were programmed using the Cycle Composer software included in the system. Sample tray was kept at 20 °C. The incubation unit was set at 40 °C and at 500 rpm shaking speed. A standard 20 ml headspace vial containing 2 ml of PFBCl 0.8% (w/v) in toluene was stored in port 2 of the incubator unit and a Na_2CO_3 2% (w/v) aqueous solution was stored in port 3, while port 1 was left free for the incubation of the different samples. The cycle begins by transferring the sample from its position in the sample tray to port 1 of the incubator and, while the sample is equilibrated, the fiber is exposed to the headspace of the reagent vial in port 2 for 10 min. Then, the fiber is moved from the reagent vial to the sample vial where it is exposed to the headspace of the sample for 15 min. After

this, the fiber is submerged 5 min in the Na_2CO_3 2% (w/v) aqueous solution stored in port 3 of the incubator. Desorption takes place by inserting for 4 min the fiber in the GC injection port, set at 250 °C. The cycle ends reconditioning the fiber by exposing it for 10 min at 250 °C under a flow of nitrogen in the bake out unit. The whole autosampler cycle is completed in 44 min; this means that a sample is completely processed per 44 min.

2.5. Method development and validation

The fiber chosen for method development was a PDMS/DVB 65 μm, which according to previous studies has a high affinity towards PFB-derivatives [20,21]. Similarly, 20 ml standard headspace vials were used in order to facilitate method automation. For the same reason, the temperatures for loading the reagent and for the further extraction-derivatization of analytes were the same. For the final tuning of the method parameters, standard aqueous solutions containing 100 mg of a API model (glycine, phenylalanine or fluvoxamine maleate) and the analytes at levels of 0.5 or 5 $\mu g\,g^{-1}$ (with respect to API) were analyzed following the general outline of the procedure but using different times and temperatures.

Validation was equally carried out in the three different models of APIs. In each case method repeatability was determined by the independent analysis of 6 APIs spiked with 0.5 $\mu g\,g^{-1}$ of AZD and CEA. Recoveries were also determined for 6 independent spiked samples (0.5 $\mu g\,g^{-1}$ of AZD and CEA) in each API model. Linearity and matrix effects were studied by analyzing 5 different concentration levels (from 0.1 to 0.6 $\mu g\,g^{-1}$ for AZD and CEA) in water and in the three API models.

A "sandwich" calibration approach was also tried. In that approach, three FVX samples containing 0.05, 0.2 and 0.55 $\mu g\,g^{-1}$ of AZD and CEA, were analyzed each between two other similar FVX standards further spiked with 0.5 additional $\mu g\,g^{-1}$ of the amines than the ones cited before; i.e. the 0.05 sample was analyzed between two FVX standards containing 0.55 $\mu g\,g^{-1}$ each one, the 0.2 between two others containing 0.7 and so on. The average signal of the two bracketing standards (analyzed immediately after and before the sample) minus the signal obtained in the analysis of the sample corresponds to 0.5 $\mu g\,g^{-1}$ of AZD and CEA and this was used as the response factor to estimate the concentration of analytes in that sample. The experiment was replicated 4 times.

Finally, in order to validate the method as screening tool, 75 samples of FVX, whose previous analysis revealed that were free of AZD and CEA, were spiked with 0.05, 0.2, 0.55, 0.7 or 1.05 $\mu g\,g^{-1}$ of AZD and CEA (15 of each concentration) and were randomly analyzed according to the proposed method in an experiment involving different days (9 days along 5 weeks), different SPME fibers (three fibers) and two different analysts. Data signals were log-transformed. Precision at each concentration level was estimated from the standard deviations of the log values. Overall precision was obtained by averaging the corresponding variances. Calibration plots were built by representing the logs of the signals vs. the logs of the concentrations and took then the form:

$$\log S = b_0 + m \log C$$

All this information was then used to build plots relating the probability of obtaining a signal higher than that corresponding to an internal specification of 0.5 $\mu g\,g^{-1}$ in the analysis of a sample of a given concentration. The operational procedure is the following:

For a sample containing C_0 concentration units of analyte, the expected average signal is obtained from the corresponding calibration graphs: $\log S_{C_0} = b_0 + m \log C_0$; similarly, for a sample containing the 0.5 $\mu g\,g^{-1}$ internally specified limit, the expected average signal is $\log S_{C_{0.5}} = b_0 + m \log C_{0.5}$

Table 1
Spectrometric properties of the PFB-amine derivatives in NCI mode.

PFB-derivative	MW	MW + PFB	Retention time (min)	NCI ions
TFEA	99	293	11.22	273 (100), 253 (15)
AZD	43	237	11.80	209 (100), 237 (76), 167 (23)
CEA	79	273	13.91	55 (100), 217 (75), 197 (65), 253 (50)
EPA	87	281	13.99	261 (100), 226 (20)

Underlined ions were used to quantify the compound.

Then, if the precision finally estimated as described above is named after σ, the z score between both signal points is:

$$z = \frac{\log S_{C_{0.5}} - \log S_{C_0}}{\sigma}$$

This represents the distance of the expected signal of the 0.5 $\mu g\,g^{-1}$ internally specified limit to the centroid of the distribution of signals obtained in the analysis of a sample containing C_0 concentration units. Hence, the probability of obtaining a signal higher than the specified limit is just the probability of having a z higher than the previous in the normal standard distribution. The procedure was applied to the determination of such probability for all concentrations below the critical limit. Above this point, a similar process was carried out but what was then computed is the probability of the signal of a sample containing more than 0.5 $\mu g\,g^{-1}$ of giving a signal below that of the expected signal of the 0.5 $\mu g\,g^{-1}$ limit.

3. Results and discussion

3.1. Method development

In the present work, volatile amines are chemically transformed by reaction with pentafluorobenzoyl chloride in order to improve the chromatographic and mass spectrometric properties of the analytes. The most important negative ions obtained in the Chemical Ionization of the PFB-derivatives of the two analytes and internal standards are shown in Table 1. As expected, the ionization technique is mild and most mass spectra are composed predominantly by less than four ions. The molecular ion of the derivative or the molecular ions minus 20 (corresponding to the neutral loss of HF) are in all cases prominent ions of the spectra, which guarantee good selectivity. The selectivity and sensitivity of the strategy can be seen in Fig. 1, which shows the ion chromatograms obtained from the different derivatized analytes in the presence of glycine, a model of hydrophilic API. Similar signals were obtained in all the APIs tested.

In order to avoid interference problems caused by the active principle, the derivatization takes place directly in the SPME fiber exposed to the headspace vapors of the sample; this approach avoids the reaction of the API with the derivatization reagent that easily occurs in liquid media – as explained in the introduction of the paper – due to its low volatility. The fiber has to contain the derivatization reagent and hence, the reproducible sorption of known amounts of derivatization reagent in the fiber was first studied. The strategy used for fixing the reagent was to expose the fiber to the headspace of a solution of the reagent in toluene, as has been previously proposed [20]. The choice of a PDMS–DVB SPME fiber was also based on results from those authors and attending to our own experience with these kinds of reagents [21]. The kinetics of the sorption of the reagent in the fiber at different temperatures was determined by directly measuring the mass of adsorbed reagent as described in the material and methods section. Results of this study are shown in Fig. 2. As can be seen, the plot reveals a logarithmic trend in which the equilibrium of the sorption process is not reached. The plot also shows that, at the low concentra-

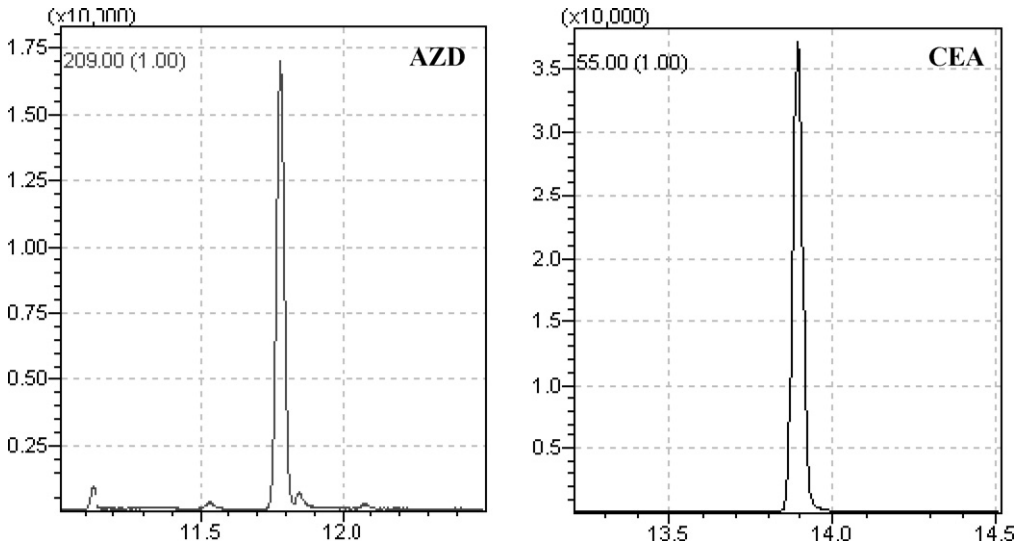

Fig. 1. Negative ion chromatograms corresponding to the PFB-amine derivatives extracted and derivatized following the proposed procedure from a GLY PAP model containing 0.5 $\mu g\,g^{-1}$ of AZD and CEA.

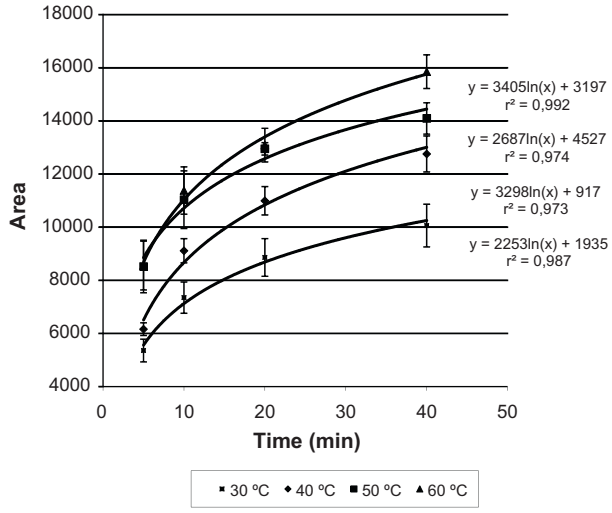

Fig. 2. Effect of time and temperature in the sorption of PFBCl in the PDMS/DVB fiber from a toluene stock solution containing 0.1% in PFBCl (w/v).

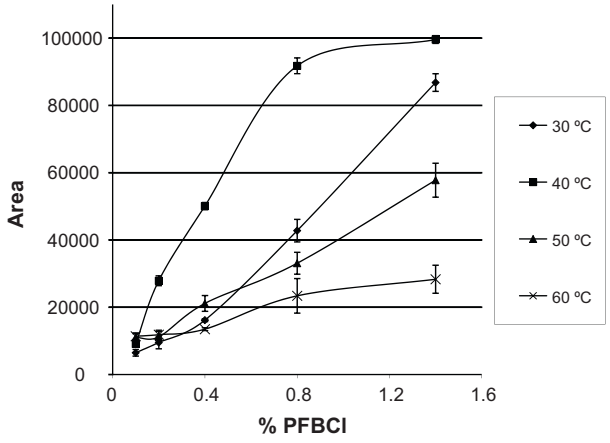

Fig. 3. Effects of temperature and of PFBCl concentration (in the toluene stock solution as %) on the sorption of PFBCl in the PDMS/DVB fiber.

tion of reagent tested in that experiment (0.1%, w/v), temperature exerts a positive effect on the sorption of the reagent, which suggests that in those conditions the sorption process is mass transfer controlled. The speed of the reagent loading process can be easily improved by increasing the concentration of the reagent in the toluene stock solution from which the head space is taken. Taking into account that the analytical method must be fast, the maximum reagent loading time was arbitrarily set at 10 min, and with this time fixed, the combined effect of temperature and concentration of reagent in the stock solution was closely examined. Results of such study are shown in Fig. 3 which shows again a marked effect of temperature, but in this case the lowest yields were observed at higher temperatures, except at the lowest concentrations of reagent. This implies that the reagent loading process becomes rather equilibrium-controlled when the presence of reagent in the gas phase is large enough. The highest mass of reagent fixed in the fiber is obtained at 40 °C from a toluene stock solution containing 0.8% of reagent, conditions that seem near to saturation, since increasing the concentration of reagent in the stock solution has no additional effect. Results at 30 °C are slightly worse, which suggest that at this temperature the mass transfer is still a problem. Finally, it should be also noted that the mass of reagent fixed when more concentrated reagent stock solutions are used are nearly an order of magnitude higher than that fixed when the concentration of the stock solution was 0.1%. For subsequent assays, the concentration of the reagent stock solution was fixed at 0.8%.

As most acid chlorides, pentafluorobenzoyl chloride is very reactive towards any substance having active hydrogen atoms, including water [22]. This made us think that perhaps the reagent could react even with the water present in the headspace of the samples, which would mean not only that water would compete for the reagent, but that the kinetics of the formation of derivatives could be strongly and negatively affected. This possibility was studied by measuring the reagent remaining in the fiber after exposing it for different times to the headspace of an aqueous solution. Results of this study are shown in Fig. 4. As can be seen, the reaction with headspace water takes place effectively (confirmed with controls subjected to the headspace of empty dry vials) and is very fast. In fact, between 70 and 95% of the reagent fixed in the fiber disappears in the first 2 min. The decay functions are power functions in which as expected, the decay is faster the higher the temperature. These observations are very important for the optimization of the method, since the use of high temperatures promotes a very fast degradation of the reagent and a compromise between reagent

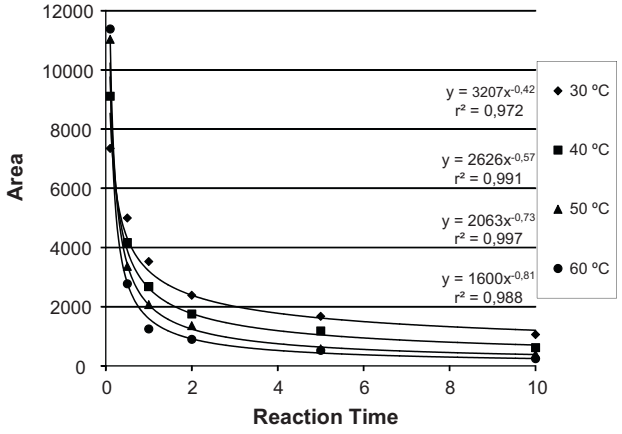

Fig. 4. Kinetics of deactivation of the reagent sorbed in the PDMS/DVB fiber upon exposition to the headspace of an aqueous alkaline solution at different temperatures.

deactivation and the transference of analytes to the fiber will have to be sought.

Finally, the high reactivity of the reagent makes it advisable to get rid of the excess of reagent once that derivatives have been formed and before the chromatographic analysis. This was easily achieved by immersing the fiber after the extraction-derivatization period in an aqueous solution containing 2% Na_2CO_3 in order to neutralize the HCl formed upon reaction of PFBCl with water. The effect of the procedure, using a 5 min deactivation time, is observed in Fig. 5. As can be seen, more than 99% of the excess of reagent is depleted during the process.

After those previous studies about the reagent, the combined effect of the temperature (the same for fixing the reagent and for further extraction-derivatization of the analytes) and of the extraction-derivatization time on the signals obtained for the analytes are shown in Fig. 6. Results are dependent on the analyte. For the lightest analyte, AZD, the best results are obtained at 30 and 40 °C, while for the CEA the best results were obtained at 40 and 60 °C, in spite of the aforementioned reagent deactivation problem. These observations may be due to the highest volatility of AZD, which would imply that at 60 °C its derivative would be nearly not adsorbed in the fiber, but a degradation of the derivative at higher temperatures cannot be completely ruled out.

The increase of signals with time in most cases also suggests that the transfer to the vapor phase of the analytes is a critical parameter of the process. Finally, a 40 °C temperature, and a 15 min extraction-derivatization process were selected as the most adequate conditions.

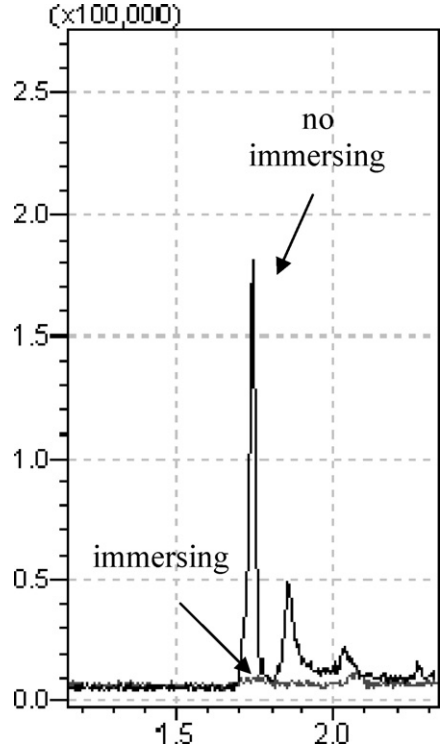

Fig. 5. Effect of submerging the fiber after the extraction-derivatization process in an alkaline aqueous solution (5 min) on the concentration of unreacted reagent.

3.2. Preliminary method validation

The method was validated in three different kinds of matrixes in order to assess its general applicability to the quantitative determination of volatile amines in different APIs. The amino acid glycine was chosen as model for highly soluble API, phenylalanine was chosen as model for hydrophobic API, and the third matrix was fluvoxamine maleate which is a real slightly hydrophobic API. The repeatability of the method obtained in the three different matrixes at 0.5 $\mu g\,g^{-1}$ level is given in Table 2. As can be seen, data are far from being satisfactory, except in the case of phenylalanine. Even in this case, method repeatability can be as high as 14% in the case of AZD. In the two other matrixes the RSD (%) figures for AZD are close to 20% and for CEA close to 15%. It is not clear why the method repeatability is better in the PHE model, and at least two explanations are plausible. On the one hand, in the water soluble cases the dissolved API would exert strong interactions with volatile amines, which would make the extraction more imprecise. On the other hand, it could be a problem of instability in alkaline medium, and the presence of a hydrophobic organic micellar suspension could

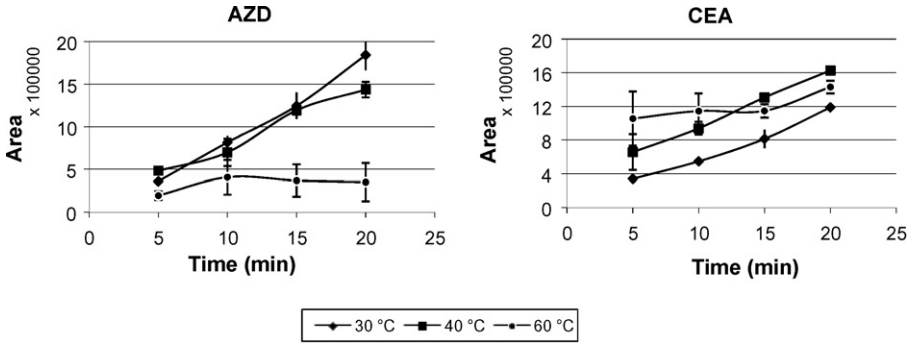

Fig. 6. Obtained signals of the analytes using different temperatures and different extraction derivatization times.

Table 2
Repeatability and recovery of the method in three different PAP models (glycine, phenylalanine and FVX).

Analyte	Repeatability (%RSD)			Recovery					
	GLY	PHE	FVX	GLY	% RSD	PHE	% RSD	FVX	% RSD
AZD	19.5[a]	14.1[a]	21.7[a]	83.4	24.5	81.8[*]	14.4	78.5[*]	25
CEA	14.0[b]	3.3[b]	17.3[b]	118.1[*]	11.1	76.1[*]	3.7	89.2	19.5

Data are peak areas normalized by that of the corresponding internal standard.
[a] TFEA.
[b] EPA.
[*] Value significantly different from 100 ($P < 0.05\%$).

help stability and therefore reproducibility. In any case, the method was unsuccessfully thoroughly evaluated in order to improve the repeatability figures. Different internal standards (not deuterated analogues) were assayed, different masses of reagent, reaction conditions and even a toluene-free way to load the reagent were also developed, but in all cases results were frustratingly similar. During all this experiment, large series of replicates were prepared and put in the autosampler tray. The signal was in no case related to the time that the sample spent waiting in the tray for being analyzed, which suggests that analyte degradation at the 20 °C at which the samples are stored is not taking place.

Four independent calibration graphs were built in the three previous matrixes and in water in order to check method linearity. Results of these experiments are given in Table 3. As can be seen, linearity for AZD is satisfactory in all cases, with determination coefficients always better than 0.99. In the case of CEA, results were slightly worse, since determination coefficients in two of the cases (water and FVX) were slightly below 0.98. As the examination of residuals did not reveal any linearity problem, it was concluded that the unexplained variance was mainly due to the method imprecision. It can be also seen in the table that the slopes obtained in the different matrixes can differ between them by factors as high as 3.4 and 1.7 for AZD and CEA, respectively, which supports the idea that the amines are exerting strong and different interactions towards the APIs dissolved or suspended in the alkaline medium. In any case, this implies that the calibration graph has to be carried out in exactly the API that is the subject of the analysis.

Method recovery data are shown in Table 2. Results given in the table correspond to the analysis of spiked samples carried out on different days and even with different SPME fibers and, therefore, they should be regarded as quite close to a real case. In all cases recoveries were not far from 100% (significant differences in 4 out of 6 cases) but are far from what should be expected for a good quantitative method. The RSD% of the recoveries are good estimates of the overall method reproducibility, and as can be seen, figures are around 25% for AZD in GLY and FVX and are just slightly better for CEA. Values are again clearly better when PHE was the matrix. It should be remarked that those reproducibility figures are just slightly worse than those of repeatability given in Table 2, which indicates that all sources of inter-batch variability are quantitatively less important than repeatability. This observation explains the failure of the highly conservative "sandwich" calibration procedure, which was also tried to improve method accuracy (see Section 2.5). In this approach, every unknown sample is analyzed between two standards prepared by spiking that sample with known amounts of analyte. The concentration of analyte in the sample is calculated using the response factor obtained in the analysis of the two bracketing standards, which should minimize matrix and inter-batch effects. Results of such experiment (data not shown) were disappointing giving again an average precision in the 20–25% range, which was not considered acceptable.

The method developed is therefore, fully automated, simple and fast, but it lacks the required precision for giving accurate estimates of the concentration levels of AZD and CEA in APIs, particularly in those soluble in alkaline water (GLY and FVX). However, the method has another interesting feature that made us to consider that yet it can be a useful analytical tool. Such feature is the low limits of detection. Method detection limits were estimated in the three different matrixes considered in this study and are shown in Table 3. As can be seen, in all the three cases detection limits are in the low $\mu g\,g^{-1}$ range, more than two orders of magnitude below the most conservative estimations derived from the established TTC of 1.5 μg [12]. For instance, for a maximum daily dosage of 300 mg of fluvoxamine maleate [23], the TTC would require a limit of detection better than $5\,\mu g\,g^{-1}$. In other APIs, such as fluoxetine hydrochloride (Prozac), maximum daily dosages are of just 80 mg [23] and hence, the limits of detection should be just better than $19\,\mu g\,g^{-1}$.

Given that most of the batches of APIs are virtually free of AZD and CEA, and that what the pharmaceutical laboratories need is a simple tool for verifying that a given batch is free of AZD and CEA, and for sending to quarantine or discarding any batch with suspiciously high levels of these compounds, a fast, simple and highly sensitive method, such as the one previously developed, can constitute an effective screening tool, in spite of its high imprecision.

3.3. Validation of the method as screening tool

A screening method, most commonly known as limit test in the pharmaceutical field, must be able to provide a simple assessment of the kind yes/no about the potential presence of the contaminant above or below the specified levels, but because of uncertainty it will also have a region of inconclusive responses. The possible answers upon the analysis of a sample containing a given level of contaminant will therefore be:

- yes, with the required level of confidence the analyte is present above the specified level (batch discarded);
- no, with the required level of confidence the analyte is present below the specified level (batch approved);
- inconclusive, it cannot be determined with the required level of confidence whether the level of the compound is above or below the specified level. In this case, the suspicious sample will have to be studied with a more accurate method or precision will have to be increased via replication.

What is therefore required in this validation process is obtaining the probability of giving a yes (or no) answer as a function of the concentration of analyte in the sample or as a function of the signal obtained in the analysis. Probability vs. concentration plots are most often used [24], and from these plots we can easily derive which are the concentration ranges at which the response will be yes, no, or inconclusive. In order to build such plots, the magnitude

Table 3
Linearity and detection limits of the method in two different PAP models (glycine, phenylalanine and FVX).

Analyte	Linearity									Detection limits ($\mu g\,g^{-1}$)		
	Slope water	R^2	Slope GLY	R^2	Slope PHE	R^2	Slope FVX	R^2	Range ($mg\,L^{-1}$)	GLY	PHE	FVX
AZD	0.00367	0.990	0.00483	0.992	0.01265	0.999	0.01052	0.992	0.1–0.6	0.002	0.001	0.003
CEA	0.02108	0.973	0.01588	0.991	0.01633	0.997	0.02757	0.978	0.1–0.6	0.003	0.003	0.002

Table 4
Log-transformed data for the calibration curves: straight lines for AZD and CEA.

C ($\mu g\,g^{-1}$)	AZD			CEA		
	log(signal)	SD	% RSD	log(signal)	SD	% RSD
0.05	3.73	0.18	4.8	3.47	0.19	5.4
0.20	4.37	0.18	4.0	4.33	0.21	4.8
0.55	4.82	0.17	3.5	4.67	0.18	3.8
0.70	4.96	0.16	3.2	4.86	0.20	4.0
1.05	5.13	0.17	3.4	5.07	0.19	3.8

	AZD			CEA		
	Slope	Intercept	R^2	Slope	Intercept	R^2
log S vs. log C	1.062	5.111	0.9997	1.186	5.042	0.993

$n = 15$.

Table 5
Concentration data (upper part of the table) and signal limits (bottom part) for the two compounds at three significance levels.

Confidence level	Limit	Concentration			
		AZD ($\mu g\,g^{-1}$)		CEA ($\mu g\,g^{-1}$)	
		$n = 1$	$n = 3$	$n = 1$	$n = 3$
95%	Lower	0.27	0.35	0.26	0.35
	Upper	0.92	0.71	0.93	0.71
99%	Lower	0.21	0.30	0.21	0.30
	Upper	1.19	0.82	1.21	0.83
99.9%	Lower	0.13	0.22	0.15	0.25
	Upper	1.57	0.97	1.61	0.98

Confidence level	Limit	Signal			
		AZD		CEA	
		$n = 1$	$n = 3$	$n = 1$	$n = 3$
95%	Lower	0.52	0.68	0.46	0.66
	Upper	1.91	1.45	2.09	1.52
99%	Lower	0.40	0.58	0.36	0.55
	Upper	2.51	1.69	2.85	1.82
99.9%	Lower	0.24	0.42	0.24	0.44
	Upper	3.37	2.02	4.00	2.22

of the signal and of its imprecision at each concentration point must be known. Although this could be derived from the validation work previously done, what we did is to analyze randomly 75 samples of AZD and CEA-free FVX spiked with 0.05, 0.2, 0.55, 0.7 or 1.05 $\mu g\,g^{-1}$ of AZD and CEA in order to have 15 replicates at each concentration. The analyses were carried out in different weeks, by different laboratory technicians and using different SPME fibers, so that they should represent a real worst-case scenario and should provide a conservative estimation of the method characteristics. The study revealed, as expected due the high imprecision, that results (chromatographic relative areas) did not follow a Gaussian distribution, but rather a log-normal distribution. Because of that, signal data were log-transformed and all the mathematic processing was done on those logarithms. Data are given in Table 4. The plots log signal vs. log concentration were in both cases two straight lines with R^2 0.9997 and 0.993 for AZD and CEA, respectively, as shown at the bottom of the table. It can also be seen that the standard deviations of the log-transformed signals were readily independent of the concentration and were also very similar for both analytes. This made it possible to calculate an averaged variance for each analyte and use it in all the studied concentration range to determine the percent of times that the signal obtained per a given concentration would be higher than that corresponding to a critical specification. The mathematical process is described in Section 2.5. The critical limit was set at 0.5 $\mu g\,g^{-1}$, which is a concentration ten times below the 5 $\mu g\,g^{-1}$ level that would correspond to the TTC at the maximum dosage for this API. The probability vs. concentration for such limit and AZD is shown in Fig. 7. As can be seen, in this case the method is able to significantly identify the presence of analyte above the internal specified level when the sample level is above 1.19 $\mu g\,g^{-1}$, and similarly is able to significantly confirm that AZD is below the specified level for samples containing less than 0.21 $\mu g\,g^{-1}$. Between these two values, results would be inconclusive for a 99% level of significance.

Results for both analytes, three significance levels and two different replication levels are given in Table 5. The upper part of the table gives results in concentration terms and the bottom part in signal terms. In signal terms these results tell us that if the signal obtained in the analysis of an unknown is below the 40% (see bottom part of Table 5) of the signal corresponding to the 0.50 $\mu g\,g^{-1}$ standard, then it can be said with 99% confidence that the level of AZD in that sample is below the specified limit. Similarly, if the signal was 2.51 times higher than that of the 0.50 $\mu g\,g^{-1}$ standard, then it can be confidently (99%) concluded that the level of AZD in the sample is above the specified level. Values in the table are reasonably good for a screening method and in fact, the practical application of this strategy to the control of AZD and CEA levels in more than 35 batches of fluvoxamine maleate has made it possible to positively confirm the absence of these compounds in 34 sam-

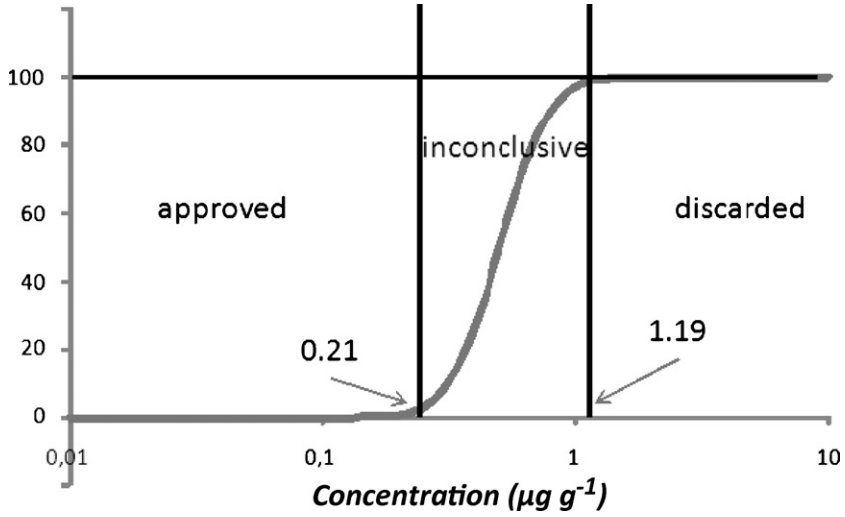

Fig. 7. Probability vs. concentration graph for the screening of AZD in PAP.

ples and to identify one as suspicious. The replicated analysis of the suspicious sample further confirmed that its levels were also below the specified limits with a 99% confidence level.

4. Conclusions

The study has demonstrated that AZD and CEA present in APIs can be sensitively determined using a fully automated analytical procedure based on the formation of PFB-derivatives on a SPME fiber exposed to the headspace of an alkaline solution or suspension of the API and further GC–NCI-MS analysis. The procedure requires nearly no manual sample handling and it is fast and convenient, and overcomes some of the limitations of previously proposed procedures. Although the analytical repeatability is not enough for an accurate quantitative determination of AZD and CEA, the method can be satisfactorily used as screening or limit test for the probabilistic-based approval or rejection of batches of API attending to the levels of AZD and CEA as it has been shown in the screening of real batches of fluvoxamine maleate.

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