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Rapid and sensitive determination of nicotine in formulations and biological fluid using micellar liquid chromatography with electrochemical detection

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ABSTRACT

Nicotine can be determined in pharmaceuticals and biological fluids by micellar liquid chromatography (MLC) using a C18 column, a mobile phase containing sodium dodecyl sulphate 0.15 M–6% (v/v) pentanol–NaH_2PO_4 0.01 M (pH 6)–KCl 0.001 M, with electrochemical detection at 0.8 V. In the optimization step, the influence of the modifiers propanol, butanol and pentanol, and the voltage has been studied. With the proposed method the analysis time is below than 8 min, linearity better than 0.999, limits of detection and quantification (ng/ml) was 4 and 12 respectively, repeatability and intermediate precision below 1.8%, and all these parameters are adequate for the quantification of nicotine in chewing gum, dermal patches, tobacco and serum samples either by a pharmacologist, pathologist or toxicologist.

1. Introduction

Nicotine is one of the most heavily used addictive substance ever available. Nicotine can enter the human body either in the form of smoke from active smokers or through passive smoking, chewing gums, sprays, inhalers, etc. Smoking is still the preferred method for nicotine intake. Pharmacologically nicotine is a compound which acts on central nervous system in form of elevation of mood, sense of euphoria and revitalizing energy but it has some very potential health hazard primary being the cardiovascular and respiratory disorder including lungs cancer [1]. Looking into its potential harm various governments all over the world have issued cautions against its use and banned its use in public places. To encourage people who wants to leave smoking various pharmaceutical companies have come up with formulation which helps the user to leave smoking and they come in the form of chewing gums, dermal patches, etc. The pharmaceutical companies as well as manufacturers of tobacco products are always in need for reliable, fast, economically and environment friendly techniques, more over when governmental agencies are up in arms against tobacco and its products. Apart form its harmful effects, studies have also been carried out to know its medicinal value with the disease like ulcerative colitis, Alzheimer's and Parkinson's diseases [2–5].

The importance of the determination of nicotine is evident from the number of scientific works carried out for the detection of nicotine in formulation [6] and products i.e. cigarette butt [7], chewing gum [8], pharmaceuticals [9], nasal powder [10], dietary nicotine [11], fertilizer [12] as well as nicotine and its metabolite in various biological samples like plasma [13–16], urine [14], hair [17,18], saliva [11], meconium [19], cutaneous nicotine [20] and milk [21] leaving apart experimental animals and animal products [22–24]. Analytical techniques for the determination of nicotine in these samples includes enzyme linked immunoassay and radio immunoassay [25,26], gas chromatography [9], gas chromatography–mass spectrometry [10,11,24], high performance liquid chromatography (HPLC) coupled with ultraviolet detector [6,12,14,15,18,20–24], diode array detection [18], electrochemical detector [17] and mass spectrometry [7,11,13,16,19].

In the biological samples, using a simple UV detection mode one may encounter other interfering substances. For this reason the method for the determination of nicotine in biological fluids needs a previous strategy prior to injection onto a chromatographic system, consisting in a pretreatment step, and the use of derivatization [27] or a specific and sensitive detector. Pretreatment step is usually performed by liquid–liquid extraction [7,9,12,13,15,17,19,21] or solid-phase extraction [14,16,18,19,23]. The extraction and derivatization methods often involve large sample volumes requiring significant quantities of chemicals and often chlorinated solvents, which result in prohibitively expensive waste storage, segregation and disposal costs. Reduction of chemicals and solvents at the source would reduce the costs and

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the time considerably. Apart from this for an extraction method to be deemed successful, both the removal of the analyte from the matrix and the trapping or concentration of the analyte prior to analysis must be optimized. On the other hand, techniques like enzyme immunoassay or radioimmunoassay has the inconvenient that the cotinine, the major metabolite of nicotine, cause interference [28]. For this reason the best strategy is the use of a detector like the electrochemical combined with the use of a chromatographic system that is warranty of specificity and sensitivity.

Micellar liquid chromatography (MLC) using mobile phases containing surfactant concentration above its critical micelle concentration (*cmc*) is an alternative method to aqueous organic HPLC because of the large number of interactions of solutes with the mobile and stationary phase. Moreover the solubilizing ability of micelles is one of their most important properties and provides direct injection of untreated samples. The most important drawback of MLC is low efficiency which can easily be improved by addition of a small amount of short chained alcohols as seen in the previous work with MLC [29,30]. This technique has demonstrated to be a useful technique in the determination of diverse groups of substances: antihistamines [31], corticosteroids [32], paracetamol [33], carbamates [34], biogenic amines [35], opiates [36], bronchodilators [37] and antidepressant [38].

The purpose of this work was to develop a MLC procedure for rapid screening and determination of nicotine in cigarette, pharmaceuticals and serum samples using a hybrid SDS-modifier mobile phase with electrochemical detection and direct injection of the samples. The method was validated according to ICH harmonized tripartite guideline [39]. This simplifies the determination of nicotine in the desired matrix. This method can be useful to analyse the compound in the fields of tobacco products and pharmaceutical quality control as well as in clinical monitorization in the de-addiction and forensic cases.

2. Experimental

2.1. Chemical and solutions

(−) Nicotine was purchased from Aldrich (Aldrich-Chemical Co. Gillingham-Dorset, England). Stock solutions containing 100 μg/ml of the compound, weighted in AX105 Delta-Range (Mettler-Toledo, Greifensee, Switzerland) were prepared in distilled-deionized water and conveniently diluted before analysis.

Micellar mobile phases were prepared by using sodium dodecyl sulphate (99% purity) of Merck (Darmstadt, Germany). The buffer salt was sodium dihydrogen phosphate, 1-propanol, 1-butanol and 1-pentanol (Merck) were used as organic modifiers. Potassium chloride (Merck) was added as electrolyte for electrochemical detection. All the solutions were filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA) and stored in the refrigerator at 4 °C.

Regarding the real samples dermal patch NiQuitin (GlaxoSmithKline, Madrid, Spain) containing 21 mg of nicotine per patch of 5 cm × 5 cm, Nicotinell Fruit Chewing gum (Norvartis, Barcelona, Spain) each chewing gum containing 2 mg of nicotine and Nicorette (Pharmacia, Barcelona, Spain) each chewing gum containing 4 mg of nicotine were purchased from the local medical store. The cigarette used were Dunhill (Dunhill, Spain) containing 1 mg nicotine per cigarette, Excite (Excite, Spain) containing 0.8 mg per cigarette. The Cigar used Dux (Dux, Spain) and Indian Cigeratte Balak Gold Filter Bidi (Balak Bidi, India) where the amount of nicotine per cigar/cigarette was not specified, were purchased from tobacco shop except for the Balak Gold Filter Bidi which was gifted by the company.

2.2. Instrumentation and apparatus

The pH of the solutions was measured with a GLP 22 (Crison, Barcelona), provided with a combined Ag/AgCl/glass electrode. The vortex shaker and sonification unit were from Selecta (Barcelona). The chromatograph was an Agilent Technologies Series 1100 (Palo Alto, CA, USA), equipped with a quaternary pump (flow-rate was 1.0 ml/min), a degasifier for the mobile phase, an autosampler (2–100 μl injection volume), finally coupled to an electrochemical detector (−400 to 1400 mV) series 1049A (Palo Alto) and an UV–vis detector (190–700 nm range). A Kromasil C18 column with 5 μm particle size, 250 mm × 4.6 mm i.d. (Scharlab, Barcelona) was used in all the experiments. Chromatographic signal were acquired and treated with the Agilent program (Revision B.03.01), and Excel (Microsoft-Office, Microsoft Corporation, Washington, USA) was used in other calculations.

2.3. Micellar liquid chromatographic method

The recommended micellar mobile phase for the determination of nicotine was SDS 0.15 M–6% (v/v) pentanol–0.01 M NaH_2PO_4 (pH 6)–0.001 M KCl. Signal with the electrochemical detector was recorded at 800 mV. For comparison, signal was also detected with UV at 259 nm. While working with electrochemical detector the main object is the maintenance of the cell surface without being polluted to have good repeatability, precision and smooth baseline. Generally frequent cleaning was not required but to maintain a smooth baseline the ED was cleaned as per the recommended procedure provided by Agilent Inc., i.e. the electrochemical cell was dismantled first, followed by cleaning the auxiliary electrode carefully with a soft tissue soaked in acetone, after cleaning the auxiliary electrode the working electrode was polished using polishing slurry provided with the electrochemical detector cell polishing kit and finally before reassembling the cell the spacers and electrode surface were cleaned with acetone using soft tissue.

2.4. Sample preparation

Chewing gum: two different form of chewing gum, i.e. sugar coated and uncoated was used. For analysis, the average weight of 10 chewing gum were determined. The chewing gum were chopped mixed with acetone and kept for extraction in an ultrasonic bath for 10 min, an accurate volume, equivalent to 10 mg of nicotine was dissolved in 100 ml of the mobile phase.

Dermal patch: 10 dermal patches were weighed, solved and extracted in acetone and finally the equivalent to 10 mg of nicotine was dissolved in 100 ml of the mobile phase.

Tobacco: regarding 10 cigarettes, the tobacco was removed and weighted, mixed with acetone and kept for extraction in an ultrasonic bath for 10 min, and after filtered and dissolved to have the material equivalent to 10 mg of nicotine in 100 ml of the mobile phase. The cigar and filter bidi where the nicotine content were not specified were also dissolved using the above technique.

Serum: blood was extracted from a smoker who smoked 8 cigarette in 2 h each cigarette containing 10 mg of nicotine. The blood was centrifuged for 10 min at 3500 rpm and the serum was diluted in a 1:10 ratio with the mobile phase prior to injection.

The aqueous samples of chewing gums, dermal patches, tobacco and serum containing nicotine were injected into the chromatographic system without any further pretreatment step than filtration through 0.45 μm Nylon membranes (Millex, Millipore Corporation, Bedford, USA), previously conditioned by passing a few ml of the recommended mobile phase.

Table 1
Optimization parameters.

SDS (M)	Modifier (%)	Retention factor (k)	Efficiency (N)	Asymmetry factor (B/A)
0.1	–	17.7	1950	2.0
0.1	4% butanol	10.1	2100	1.8
0.1	4% pentanol	4	2500	1.6
0.15	6% pentanol	2.54	2800	1.35

2.5. Method validation

Method validation was performed to meet the criteria specified by ICH guideline [39]. Linearity and sensitivity were checked by injecting a series of the analyte at different concentration levels in order to cover the whole working range. Calibration curves of the spiked samples for nicotine were calculated by a least squares linear regression analysis by plotting the peak area of each analyte versus the analyte concentration. The limit of detection (LOD) and the limit of quantification (LOQ) was based on the *3s criterion* and *10s criterion* using a series of 10 solutions containing a low concentration of the compound.

Accuracy was also included in the validation procedure: intra-day ($n = 6$) and inter-day ($n = 5$) precision. Accuracy and precision were studied by analysing three different concentration levels corresponding to 125, 250 and 500 ng/ml.

3. Result and discussion

3.1. Optimization of the mobile phase

Experimental work was focused on the optimization of the conditions for the simple and rapid, as well as low cost and not time consuming analysis including the selection of proper mobile phase to obtain satisfactory results. The optimization of the method was carried out by using different mobile phases.

3.1.1. Surfactant concentration

The effect of SDS concentration on the separation of nicotine was studied using mobile phases containing SDS in concentrations 0.05, 0.1 and 0.15 M. As is usually observed in MLC, for nicotine also when increase the concentration of SDS, decrease the retention time. In SDS 0.05–0.01 M NaH_2PO_4 (pH 6)–0.001 M KCl the retention factor recorded is higher than 28. This value is reduced in SDS 0.10–0.01 M NaH_2PO_4 (pH 6)–0.001 M KCl to around 17, but in SDS 0.15–0.01 M NaH_2PO_4 (pH 6)–0.001 M KCl the retention factor is of 10, too higher to obtain a rapid method. In the three mobile phases there was no marked change in the efficiency which was around 2100.

3.1.2. Modifier concentration

The next step was to see the effect of modifier on various chromatographic parameter where the surfactant SDS used for all the analysis was kept fixed at 0.10–0.01 M NaH_2PO_4 (pH 6)–0.001 M KCl, with 4% (v/v) of different organic modifiers (propanol, butanol, pentanol). In the individual chromatograms obtained for the analyte in these mobile phases the retention factor (k), efficiency (N) and asymmetry factor (B/A) were measured. The dead volume was determined as the mean value of the first significant deviation of the baseline in the chromatograms of the analytes. Several mobile phases were used in order to find de optimum composition of the mobile phase. Results obtained are summarized in Table 1 which shows that the retention factors decrease for SDS and alcohol while increasing the concentration of both. Finally, increasing the concentration of SDS to 0.15 M and pentanol 6% (v/v) retention factor decreased by around 40%. Regarding the efficiency was the highest, and B/A was adequate for the quantification of nicotine. Thus we can conclude form the above study that the solvent strength in MLC depends on the organic modifier nature. In particular for the alcohols, the longer the alkyl chain of the alcohol, the larger the solvent strength. In other words, as the length of the alkyl chain of the alcohol increases, the interaction with the solutes is stronger and the alcohol can compete efficiently with micelles [30].

Table 2
Calibration parameters and limits of detection (LOD in ng/ml, *3s criterion* and LOQ in ng/ml, *10s criterium*) for the determination of nicotine using the optimized MLC method (150 mM SDS–6% (v/v) pentanol (pH 6)).

Detector	Slope	Intercept	r^2	LOD	LOQ
UV	33.04 ± 0.03	−0.46 ± 0.06	0.9999	4	12
ED	373.56 ± 0.07	−1.4 ± 0.3	0.9999	0.17	0.4

3.1.3. Study of the pH

To study the effect of pH on area, retention factor and efficiency, 10 injections were made using a mobile phase (0.15 M SDS–0.01 M NaH_2PO_4–0.001 M KCl–6% (v/v) pentanol) buffered to pH 3, pH 6 and pH 7. The study showed that pH had no marked change either in the area or on the efficiency for nicotine but the retention factor showed a change from 6.3 using pH 3, to 2.6 at pH 6 and 2.3 while using pH 7. The best time was achieved using a mobile phase buffered to pH 7 but the background noise was higher when the potential was raised from 700 to 800 mV. Thus a mobile phase buffered to pH 6 was studied there was not a marked change in the retention factor compared to pH 7 but reduction in the background was noted when the electrochemical detector was adjusted to 800 mV. A mobile phase buffered to pH 6 is also column friendly, eco-friendly and more over there was less background problem using electrochemical detector as compare to pH range above 7, so it was decided to carry out further study using pH 6.

While using UV detection with direct injection of biological sample, fast eluting peak overlap with the tailing of the protein band, but this factor is not so effective while using electrochemical detector. For this purpose we have decided to work with maximum concentration of SDS and pentanol, the longest chain modifier, to speed up the separation without the loss of the sensitivity. Thus the optimum mobile phase was SDS 0.15–0.01 M NaH_2PO_4 (pH 6)–0.001 M KCl–6% (v/v) pentanol.

3.1.4. Oxidation potential

In order to establish the oxidation potential that gives maximum peak area for the detection nicotine, the applied potential was varied for 200–900 mV, in intervals of 50 mV. At each voltage, 10 injections were made and the peak area was measured. The compound starts to be oxidised at potential higher than 550 mV, and the peak area does not show marked change beyond 900 mV range. In this work while working with biological and pharmaceutical matrix without any pre-treatment, it was convenient to work at low potential, without the loss of the sensitivity. Therefore for this work a working potential of 800 mV was used.

3.2. Method validation

The proposed method was validated in compliance with ICH guideline [39]. The following parameters were validated.

3.2.1. Linearity

Calibration curves were constructed for nicotine, using the measured areas of the chromatographic peaks at six increasing concentrations in the range from 0.03 to 2 μg/ml in micellar medium and serum samples. The slopes, intercepts and regression coefficients of the calibration curves are given in Table 2. Limit of detection was calculated using signal-to-noise approach as per

Table 3
Intra and inter-day precision (C.V., %) using three different concentrations, of nicotine (c_1 = 500, c_2 = 250 and c_3 = 125 ng/ml) and the optimized MLC-ED method applied to serum samples.

Sample	Intra-day (n = 6)			Inter-day (n = 5)		
	c_1	c_2	c_3	c_1	c_2	c_3
Serum	0.76	1.07	1.83	0.55	0.85	1.19

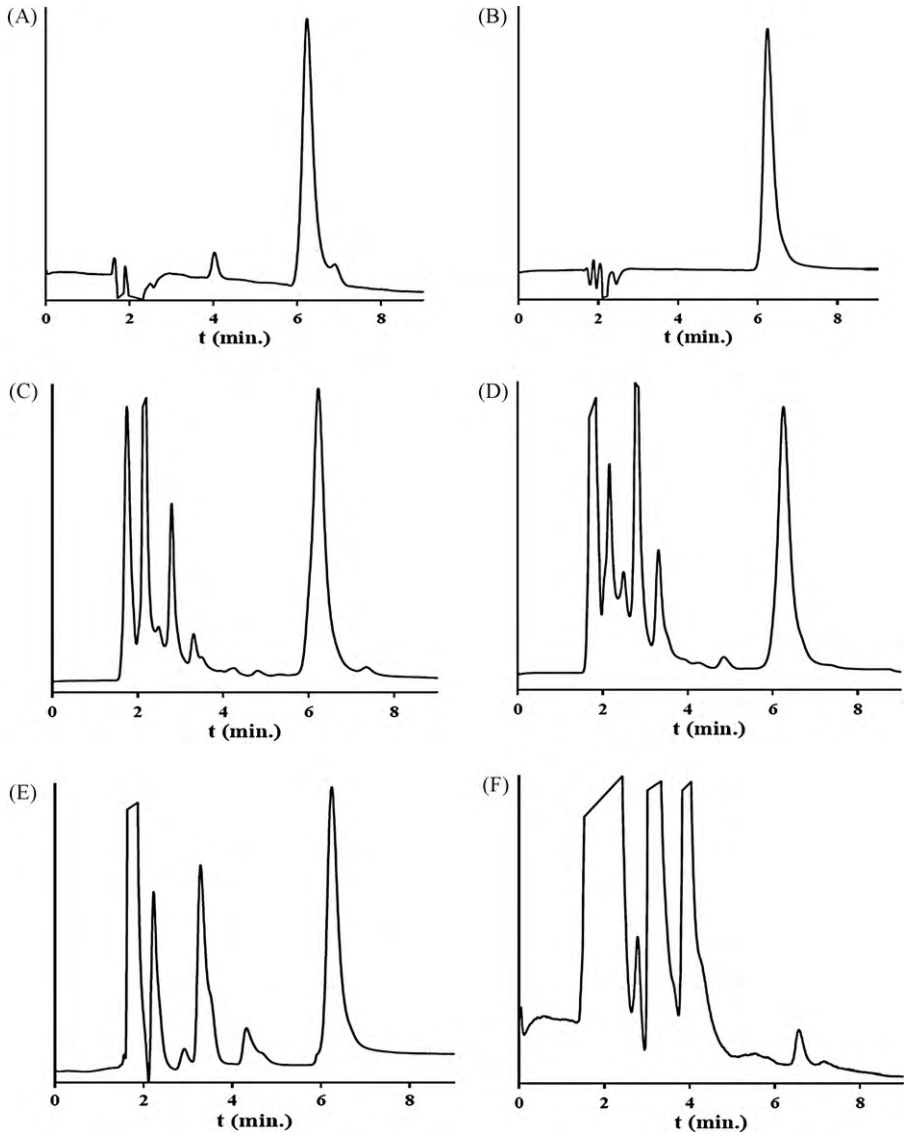

Fig. 1. (A and B) shows the chromatogram obtained after injecting extract of chewing gum and dermal patch, while (C and D) shows the chromatogram obtained after injecting extract of balak gold filter bidi and Dunhill cigarette respectively. (E) Shows spiked (1 μg/ml) serum sample where as (F) shows a real serum sample diluted 10 times of a smoker volunteer. Applied potential: −800 mV. Mobile phase: 0.15 M SDS, 6% (v/v) pentanol, pH 6.

Table 4
Determination of nicotine in tobacco and pharmaceuticals using the MLC-ED method.

	Composition	Label claim (%)	RSD (%) (n = 5)
Pharmaceutical			
Nicotinell Fruit Chewing gum	2 mg of nicotine per chewing gum	93	0.9
Nicorette Chewing gum	4 mg of nicotine per chewing gum	91	0.7
NiQuitin Dermal Patch	21 mg of nicotine per patch of 5 cm × 5 cm	97	1.05
Tobacco product			
Excite	0.8 mg per cigarette	105	1.2
Dunhill	1 mg per cigarette	109	1.6

recommended by ICH Harmonised Tripartite Guideline [39] for validation of analytical procedure where determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be detected. A signal-to-noise ratio of 3 was considered for calculating the detection limit. For calculating the limit of quantification the same procedure as of above was repeated but in this case a signal-to-noise ratio of 10 was considered as could be seen in Table 2 and permitted the quantification of the nicotine in cigarette, pharmaceuticals and serum samples.

3.2.2. Intra- and inter-day precision

Intra-day precision (Table 3) for nicotine in micellar media and serum samples was calculated by measuring the areas of the peaks obtained from the six injections of three test solutions on the same day. The intra-day precision was determined at three different concentrations within the calibration ranges of 125, 250, 500 ng/ml.

Inter-day precision was calculated by measuring the area of the peaks obtained from five injections of three test solutions of nicotine in micellar media and serum samples (two injections for test solution) on tenth day over a three-month period and made by different analyst's and equipment, at three different concentrations. Table 3 shows calculated inter-day precision, (%, C.V.), which was always below 2%.

3.2.3. Analyses of nicotine pharmaceutical formulations

Once the conditions for separation and quantification were established, the MLC method was applied to different pharmaceutical formulations (chewing gum and dermal patch) containing nicotine and different tobacco products in the form of cigarette (dunhill, excite), bidi (balak bidi) and cigar (dux). The pharmaceutical and tobacco product extract was injected directly on to the MLC system without any pre-treatment and the results are shown in Table 4. Fig. 1A and B shows the chromatogram obtained after injecting extract of chewing gum and dermal patch while Fig. 1C and D shows the chromatogram obtained after injecting extract of balak gold filter bidi and Dunhill cigarette, respectively.

3.2.4. Analyses of nicotine in biological samples

The applicability of the method for the determination of nicotine was verified by its determination in serum samples. The background signal of serum, due to proteins (wide band at the head of the chromatograms) and several endogenous compounds (peaks at diverse retention times) can seriously affect identification of fast eluting compound using direct injection with using high oxidation potential. Direct injection of spiked serum samples (without any dilution) with electrochemical detector using potential below 600 mV does not show any problem due to high oxidation potential required by endogenous compounds present in the serum. But in this case as the oxidation potential was 800 mV we can see endogenous compounds oxidising until 4 min. But as the elution time of nicotine was more than 6 min these endogenous oxidising compounds do not have any negative effect on the efficiency of nicotine analysis. In this study the serum sample was diluted 10 times, due to the fact that injection of a large number of undiluted serum samples can produce damage to the column and moreover the nicotine in the sample can be detected at this dilution therefore it was decided to carry out the analysis of serum samples after their dilution in the ratio of 1:10. Fig. 1E shows the chromatogram of serum spiked with 1 μg/ml nicotine and Fig. 1F shows the chromatogram of serum sample of one volunteer who kindly donated blood sample for experimental purpose only.

The results indicate that the MLC procedure can be easily used for the determination of nicotine in cigarette and pharmaceutical samples with analysis time below 8 min using a mobile phase of 0.15 M SDS–0.01 M NaH_2PO_4–0.001 M KCl–6% (v/v) pentanol at pH 6 coupled to an electrochemical detector. The procedure can also easily be implemented for its monitorization of nicotine in serum samples at the normal and toxicological levels, and also in pharmaceutical and cigarette quality control taking into account that different matrix were injected without any previous treatment except for filtration and dilution.

4. Conclusions

The method developed here has the major advantage of direct injection of biological as well as pharmaceutical or commercial samples by solublizing the component in desired solvent. The addition of alcohols to the micellar phase can result in an additional interaction with the solute. The variety of possible interactions gives a large versatility to this technique as an alternative to conventional HPLC and makes it appropriate for a wide range of solute analysis. Another advantage is the low amount or organic solvents used which reduces toxicity, flammability, environmental impact and cost of these phases. The detection limit achieved is lower than the reference methods as well as the analysis time is lower.

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