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Abstract
Honey is a food produced from honey bee widely used for the sweetening power and for its biological properties. In order to prevent the infection of the hive, different xenobiotics (antibiotics, pesticide) were frequently employed. One of these substances is the chloramphenicol, that given its chemical stability could often found in food. Chloramphenicol have several side effects in humans after their ingestion and for this reason their intake must be avoid. The aim of this study, was developed an ultrasound-assisted dispersive liquid-liquid microextraction method coupled with UHPLC MS/MS determination, for fast and accurate analysis of chloramphenicol in honey. The parameters affecting on extraction efficiency were carefully optimized using an experimental design in order to maximized the recovery reducing matrix effects. After the optimization the method was validated and successfully applied to 66 honey samples.
Graphical abstract

Keywords: Honey Chloramphenicol quantitative analysis UA-DLLME, Validation Reg. 2002/657 EC Experimental design optimization, UHPLC MS/MS

1. Introduction

In nature there are many contaminant, both inorganic and organic as mycotoxins (Zinedine et al. 2010), pesticide (Seccia, Albrizio, Fidente, & Montesano 2011; Seccia, Fidente, Montesano, & Morrica 2008), bisphenol (Grumetto, Montesano, Seccia, Albrizio, & Barbato 2008) that have been be found in foods. Antibiotics are another class widespread used and found in foods such as milk meat fish and honey (Bishop & White 1984; Johnson, Jadon, Mathur, & Agarwal 2010; Kennedy, McCracken, Cannavan, & Hewitt 1998; Lee, Lee, & Ryu 2001).
Honey is a food produced from honey bee, mainly constitute from glucose and fructose for about 75% of the total weight and due to this composition has always been used as a natural sweetener or as sugar substitute (da Silva, Gauche, Gonzaga, Costa, & Fett 2016). In addition to sugar parts, honey contains a complex mixture of several organic constituents, such as vitamins, di/tri/oligo saccharides, amino acids, proteins, and inorganic constituents including water, magnesium, calcium, manganese, potassium, sodium, copper, iron, chloride, phosphorus etc. Moreover, honey include other minor constituents as polyphenols (flavonoids and phenolic acids), carotenoid-like compounds, ascorbic acid, Maillard reaction products, that are related to its wide biological activities (Campone et al. 2014; da Silva et al. 2016). The beehives are attacked by numerous parasites and pathogens such as microsporidia, fungi, viruses, and bacteria (Allen & Ball 1996; Chen, Pettis, & Feldlaufer 2005; Evans & Schwarz 2011; McMenamin & Genersch 2015). The most widespread infectious brood diseases, that effects honey bee larvae are the American and Europe foulbrood (Krongdang, Evans, Chen, Mookhploy, & Chantawannakul 2018). American foulbrood (AFB) is an infectious caused by the spore-forming bacterium *Bacillus* larvae (Hansen & Brødsgaard 1999) whereas the European foulbrood (EFB) is caused by *Melissococcus plutonius*, (Forsgren 2010) a bacterium that infects the

midgut of bee larvae. As a consequence, in order to prevent or to cure the beehive infections, various treatments have been considered including the use of xenobiotics. Unfortunately, the incorrect use of antibiotics may leave residues in foods (Landers, Cohen, Wittum, & Larson 2012; Lee et al. 2001). These antibiotic residues, even if at trace levels, may have direct toxic effects on consumers e.g., allergic reactions or indirectly, causing the induction of antibiotic resistant strains of bacteria. Among the various antibiotics available, chloramphenicol (CAP) has been widely used in veterinary medicine, for its broad-spectrum activity against broad variety of pathogens. Chloramphenicol was originally isolated from the bacterium *Streptomyces venezuelae* (He, Magarvey, Piraee, & Vining 2001) which is effective against a wide range of Gram positive and Gram negative bacteria (Vivekanandan, Swamy, Prasad, & Mukherjee 2005). The potentially fatal side-effects such as aplastic anaemia (Balbi 2004; Kleiman & Abel 2018) and the suspected carcinogenetic effect, led the International Agency for Research on Cancer (IARC) considered as probably carcinogenic to humans and to assigned CAP in group 2A. Due this high toxicity, that are dose independent, CAP has been banned for use in food producing animals in many countries including EU. As a consequence, CAP is included in Annex IV of Council Decision 2377/90 (Regulation, H. A. T 1990) which list all substances with zero tolerance in food of animal origin. As result of strict regulations regarding the use of CAP in various food products fast, reliable, sensitive and accurate method are necessary, in order to minimize the public health problems. In the European community (EC), the method to analyse drug with an establish zero-tolerance level the must meet the requirements of confirmatory methods fixed in EU decision 2002/657/EC (Commission Decision 2002/657/EC 2002). In particular, regarding the determination of chloramphenicol in all food of animal origin including honey, a minimum required performance limit (MRPL) of 0,3 μg kg^{-1} was lay down by the EC. Currently the analysis of CAP residue in food is carried out using several rapid screening methods and subsequently in case of positive samples, the results must be confirmed by a confirmatory method. However, despite these screening methods generally provide a semi-quantitative estimation of total residues detected and provide a high number of false positive (Gaudin, Cadieu, & Maris 2002) they still continue to be used for their simplicity and low cost. Nowadays, with the continuous growth of samples to analysed, there is a constant requirement for more accurate, simpler and faster analytical methods for the quantification of contaminants in food, in order to reduce time and cost and increase the samples throughput. Given the chemical complexity of food matrices with the presence of many potential interferences, specific and selective methods are requiring. Mass spectrometry determination is currently recognized as analytical tools capable to provide unambiguous identification and accurate quantification of trace contaminants in food samples. Nevertheless, despite the high selectivity and sensitivity of LC-MS/MS methods, this technique, does not provide the performances required by regulatory and food safety agencies. Therefore, a sample preparation technique, able to remove matrix interferents and concentrate the analyte must be perform prior the analytes determination. The most common sample preparation techniques used prior the veterinary drug determination are solid-phase extraction (SPE), liquid–liquid extraction (LLE), pressurized liquid extraction (PLE), matrix solid phase dispersion (MSPD) (Stolker & Brinkman 2005). Although these clean-up techniques are able to remove many of the matrix interferences allowing more sensitive and accurate LC–MS/MS analysis, they have few drawback, for example are tedious, does not able to obtain high enrichment factors, require an extensive use of organic or petroleum derivatives solvents, producing a negative impact on the ecosystem health. Therefore, it is important to develop novel, simple and quick methods that reduce as much as possible the main restrictions of the traditional procedures; providing reliable and accurate result, able to meet the current guidelines set by EC. Over the recent years, significant advances have been made in order to make analytical procedure simpler, rapid and eco-friendly, keeping at the same time high selectivity and sensibility (Campone et al., 2014, Campone et al., 2015, Campone et al., 2016). In this perspective, dispersive liquid–liquid microextraction (DLLME) can be considered an interesting non-conventional sample preparation method to be used as an alternative to classical methods. DLLME has been presented for the first time in 2006 by Rezaee et al. (2006), it is based on a binary solvent mixture (extranet

dispersant) able to extract the analytes from aqueous samples. In this technique, the extraction of analytes is performed by a cloud of fine extraction solvent drops, which is formed when an appropriate mixture of solvents is injected in the aqueous samples. Since the first application to date, DLLME have been widely used on a broad range of samples and analytes (Campone et al. 2014; Campone, Piccinelli, Celano, & Rastrelli 2012; Campone, Piccinelli, Celano, Russo, & Rastrelli 2013; Campone, Piccinelli, & Rastrelli 2011) because it is extremely simple, quick, efficient, low solvents consumption and not require particular expensive equipment. In order to improve the performance of this technique and extend its application on a large range of analyte and matrices, several modifications have been developed (Ahmad, Al-Sibaai, Bashammakh, Alwael, & El-Shahawi 2015; Rezaee, Yamini, & Faraji 2010). One modification of DLLME technique is called ultrasound-assisted dispersive liquid–liquid microextraction (UA-DLLME), where an ultrasound wave is applied after the injection of extraction/dispersant mixture to promote the formation of the fine cloudy droplet. The sonication step is able to increase the extraction efficiency and reduce the equilibrium time.
The aim of this work was to develop a new UA-DLLME method for the rapid extraction and accurate determination of CAP in honey. The main UA-DLLME conditions affecting on extraction efficiency such as volume of extraction/dispersant solvent and salt addition were carefully investigated and optimized by using an experimental design. Subsequently, under optimized condition the method was validated and finally applied to 66 honey (Italian and imported) from different botanical origins. To the best of our knowledge, this report describes the first application of UA-DLLME to the determination of CAP in honey.

2. Experimental

2.1. Chemicals and reagents

Chloramphenicol standard (CAP) purity ≥98%, was purchased by Sigma-Aldrich (Milan, Italy), stock solution of CAP (100 ng mL^{-1}) used for spiking procedure and to obtain calibration curve and matrix matched calibration curve was prepared in acetonitrile (ACN) and stored in glass vials at 4 °C for a maximum of one week. Ultrapure water (18 MΩ) was obtained by a Milli-Q system (Millipore, Bedford, USA). Analytical grade acetone (Me_2CO), acetonitrile, methanol (MeOH), chlorobenzene (C_6H_5Cl), chloroform ($CHCl_3$), 1,2 dichloroethane ($C_2H_4Cl_2$), and dichloromethane (CH_2Cl_2), 1,4-Dibromobutane ($Br(CH2)_4Br$) and sodium chloride (NaCl), were purchased from Sigma-Aldrich (Milan, Italy). MS-grade water (H_2O), and methanol (MeOH), were supplied by Romil (Cambridge, UK). MS-grade Formic acid (HCOOH) and Ammonium formate (AF) purity ≥99% (HCO_2NH_4) were supplied by Sigma-Aldrich (Milan, Italy).

2.2. Honey samples

Honey samples were obtained from beekeepers, or purchased in markets of Campania region (Italy). A mixture of three non-contaminated honeys samples, from different botanical origin (acacia, chestnut and citrus honey), were combined and homogenized using a blender (Ultra-Turrax T25, Ika® Werke GMBH & Co. KG, Staufen, Germany) in order to obtain a representative blank matrix. Before the extraction 1.0 g of pooled honey mixture was weighed into a 15 mL centrifuge tube with conical bottom, diluted with 5.0 mL of water (15% *w*/*v* NaCl) and vortexed until a homogeneous sample was obtained. During the method development, honey solution was fortified by adding an appropriate volume of standard and left to equilibrate for 1 h under stirring. The pooled blank and spiked samples were used in the optimization and validation of UA-DLLME procedure.

2.3. Ultrasound-assisted liquid-liquid microextraction for the extraction of chloramphenicol

The chloramphenicol extraction from honey was performed by ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME). Briefly, an aliquot of 5.0 mL of homogeneous honey solution (0.2 g mL^{-1}) was placed in a 15 mL polypropylene test tube with conical bottom. The binary mixture composed to 800 µL of ACN (dispersive solvent) and 300 µL of $CHCl_3$ (extraction solvent) was

rapidly injected into the sample by using a glass syringe. Then, the test tube was gently shaken by hands for several seconds and immerse in the ultrasounds bath for 2 min. Due to the combined effect of shaking and sonication a stable cloudy solution was formed in the test tube and in this moment the analyte was extracted into the fine droplets of extraction solvent. Subsequently, sample was centrifuged (ALC centrifuge PK 120, Thermo Electro Corporation, San Jose, CA, USA) for 5 min at 13000 rpm and the extraction solvent with a high density than water, settles on the bottom of conical tube. Before recovery the extraction solvent ($CHCl_3$) by a HPLC glass microsyringe, most of aqueous supernatant was removed using a Pasteur pipette. The extraction solvent was transfer into 2 mL plastic tube and dried under a gentle nitrogen flow, the residue was reconstituted with 100 µL MeOH/H_2O 3:7 (*v*/v) and finally injected into HPLC system. All the experiments were performed in triplicate.

2.4. UHPLC-MS/MS method

The quantitative analysis of chloramphenicol in honey was performed on a Shimadzu Nexera UHPLC system (Shimadzu, Milano, Italy), consisting of a CBM-20A controller, two LC-30 CE dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, a CTO-20A column oven, and a SIL-30 AC autosampler. The UHPLC system was interfaced with an API-6500 triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with a TurboIonSpray® source operating in negative ion mode. The extracts were chromatographed on a Kinetex C18, UHPLC column (50 × 2.1 mm, 1.7 µm; Phenomenex, Bologna, Italy), using H_2O (A) and MeOH (B) as mobile phases. After injection (10 µL) the analyte was eluted using the following gradient: 0–0.5 min, 5% B, 0.5–1.0 min, linear increase to 20% B, 1.0–3.0 min, linear increase to 40% B hold of 1.0 min, 4.0–7.0, linear increase from 40 to 98%B. The column was kept at 30 °C and the flow rate was set at 0.3 mL min^{-1} for all the chromatographic run. At the end of each run the column was washed with 98% B to remove the matrix interferents, and re-equilibrated with 5% B for 6 and 5 min respectively. Analyst™ software version 1.6 (AB Sciex, Toronto, Canada) was used for mass spectrometer control and data acquisition/processing.

In order to improve the analyte ionization and to select the multiple reaction monitoring (MRM) transition, tune optimization was carried out by the direct infusion of CAP standard solution at concentration of 5 µg mL^{-1}. The optimized ion source parameters were as follow: ion spray voltage (IS) -4500 V, source temperature (TEM) 400 °C, Dwell time was 50 ms for each MRM transition, nebulizer gas (GS1) 45 psi, heater gas (GS2) 30 psi, curtain gas (CUR) 35 psi, collision gas (CAD) medium. Nitrogen was used for both nebulizer and collision gas and collision energies was optimized for each analyte transition during infusion of the pure standard. For the proposed method, the most intense transitions and one characteristic ion were chosen for quantification and confirmation of analyte respectively. In order to satisfy the qualitative system of identification points (IPs), according to the Commission Decision 2002/657/EC criteria for prohibited substances for analyte quantification the precursor/product of ^{35}Cl-CAP ion selected were *m/z* 320.9 → 256.9 (CE = −14) whereas for analyte identification one product ion was used m/z 322.7 → 256.9 (CE = −16) of ^{37}Cl-CAP ion.

2.5. Experimental design

The experimental designs and statistical analysis were estimated using Statgraphic Centurion XVI Version 16.1 (Rockville, USA). The effect of three independent factors on extraction efficiency and matrix effect of UA-DLLME were studied by an experimental design and the ranges within each factor, was selected by preliminary experiments. Specifically, a response surface Box–Behnken design 2-factor interactions was carried out considering, three variables at low, medium and high levels: volume of extraction solvent at 100, 200 and 300 µL, volume of dispersant solvent 600, 700 and 800 µL and amount of NaCl in honey solution (NaCl %) 5, 10 and 15%.

Two response variables were individually considered in the optimization of the extraction conditions: peak area (PA) of CAP spiked into the samples; matrix effect (ME) calculated by comparing the PA of CAP obtained injected a standard solution and that from a post-extraction spiked sample. A total of 2 block of 15 experiments for each block (12 points of the factorial design, 3 center points, 5

freedom degree) were carried out in randomized order. The optimum experimental conditions that independently maximized PA and minimized ME, were obtained from the fitted model. Analysis of variance (ANOVA) Fisher's least significant difference (LSD) procedure were carried out to made a multiple comparison procedure in order to determine which means are significantly different from which others. The experimental matrix design, with the experimental range for factors and the results obtained for the analysed response variables were shows in Table 1S.

2.6. Method performances and matrix effects evaluation

The validation procedure followed the decision 2002/657/EC with some modification in order to find a good compromise with practical aspects of laboratory work.

Linearity was evaluated through the regression coefficient (R^2) of calibration curve prepared by diluting appropriate volumes of CAP standard solution with H_2O:MeOH (7:3 *v*/v) (solvent curve) or with UA-DLLME extract of honey CAP-free (matrix-matched curves). The linearity of the solvent and the corresponding matrix-matched curves were evaluated in the working range of 0.1–5 ng mL^{-1} (five calibration levels). The statistical analysis of variance (ANOVA) was performed to determine the goodness-of-fit and linearity of the curves.

Selectivity of proposed method was assessed through the average (μN) and standard deviation (σ N) of the noise amplitude of blank samples, moreover the intercept of calibration curve was set as (μN). Furthermore, the decision limit CCα defined in 2002/657/EC as "the concentration of an analyte at and above which a sample is really non-compliant with an error probability α" (<1% for prohibited substances) was calculated by the following equation.(1)

where (σN) and (*a*) are the standard deviation of noise of the noise amplitude of blank samples and the slope of calibration curve respectively.

The detection capability CCβ defined in 2002/657/EC as "minimum amount of a compound that can be detected, identified and/or quantified with an error probability β (<5% for all substances) was also calculated following the Eq. (2).(2)

where (σS) and (*a*) are standard deviation of the signal amplitude of spiked samples at [S/N = 6] and the slope of calibration curve respectively.

Recoveries studies were evaluated by spiking blank pooled honey sample with standard of CAP at three different contaminations levels 0.1, 0.3 and 1 ng g^{-1}.

In order to determine the matrix effect phenomena (signal suppression or enhancement), slope of solvent and matrix-matched curves were compared. Repeatability was estimated from the analysis of five independent extraction of spiked honey samples through the relative standard deviation (RDS%) of the signal amplitude. Considering that matrix effect phenomena was not observed, to quantify CAP in real samples solvent calibration curve of quantify ion (*m*/*z* 320.9 → 256.9) was used.

3. Results and discussion

In residue analysis, especially when the concentration of analyte is at trace level, the crucial step and also the bottleneck of the whole procedure is the sample clean up. The selection of sample preparation technique is usually carried out on the basis of two main characteristics: i) its ability to remove interfering compounds; ii) and the capability to pre-concentrate the analyte. For this reasons, a sample preparation easy, fast and sensitive should be used in order to improve the extraction efficiency and increase the sample throughput. Since the high chemical variability of honey, the optimization of extraction conditions was performed using a mixture of three non-contaminate honey belonging to different botanical origin (acacia, chestnut and citrus), in order to obtain a representative sample, more complex than using a single variety of honey.

3.1. Optimization of UA-DLLME parameters

3.1.1. Preliminary experiments

At beginning the comparison between DLLME and UA-DLLME was carried out in order to select the more appropriate extraction technique. The starting condition of extraction procedure applied to compare these two procedure were selected on the basis of our experience in DLLME analysis and were as follow: 5 mL of spiked (1 μg Kg^{-1}) honey solution (0.2 g mL^{-1}), 600 μL ACN and 200 μL of CH_2Cl_2. These conditions were used both for the classic approach of DLLME as well as UA-DLLME. The only difference between these two techniques, was that in the UA-DLLME, after the injection of the extraction/dispersive mixture into the honey aqueous solution, the sample was sonicated for 2 min. The results showed that, UA-DLLME was more effective in the extraction of CAP than DLLME probably because when sample was sonicated, the formation of the cloudy state was promoted, resulting in a decrease of the MS/MS signal variability and also in a slightly increase of the extraction efficiency (data not show). This may be due to a greater rate of transfer of analyte when ultra-sonication was carried out, thus in all the further experiments a sonication step of 2 min was carried out immediately after the injection of the extraction/dispersant solvent mixture. Once selected the better extraction technique, the influence of several parameters on the UA-DLLME extraction efficiency such as nature of extraction and dispersive solvent, pH of honey sample solution, and effect of salting out were preliminary investigate before performing their optimization by the experiment design. This strategy was preferred over the use of an experimental screening design based on our previous experiences with the optimization and development of DLLME methods.

In order to evaluate the influence of each parameter on extraction efficiency, MS/MS normalized peak area of CAP was selected as response variable. First of all, the main two parameters that have an influence on the UA-DLLME extraction efficiency, type of extraction and dispersant solvent, were evaluated. A good extraction solvent employed in a DLLME process must possess, (i) higher density than water, (ii) high affinity with target compounds, (iii) low solubility in water, (iv) and produce a stable cloudy solution (fine dispersion of insoluble solvent extraction in water) in the presence of a dispersive solvent when injected into aqueous solution. Among the solvents with these characteristics, chlorinated and brominated solvents, $Br(CH_2)_4Br$ (2.17 g mL^{-1}), CH_2Cl_2 (1.32 g mL^{-1}), $CHCl_3$ (1.47 g mL^{-1}), $C_2H_2Cl_2$ (1.25 g mL^{-1}) and C_6H_5Cl (1.11 g mL^{-1}) were evaluated. The experiments were performed by using a mixture of 600 μL of ACN as dispersive solvent and 200 μL of each extraction solvent. In the case of $Br(CH_2)_4Br$, a stable cloudy solution was not observed and as a consequence low peak area response was obtained (Fig. 1A). For all the other tested solvents, a cloudy state was obtained and as shown in Fig. 1A, C_6H_5Cl gave lowest response than $CHCl_3$ CH_2Cl_2 and $C_2H_2Cl_2$; between these solvents, $CHCl_3$, provided clearest sediment phase, so it was selected as extraction solvent in the further experiments.

The selection of the dispersing solvent must be carried out considering the miscibility both with the extraction solvent and aqueous sample. Therefore, on the basis of these requisites the choices were limited to the methanol, acetonitrile and acetone. Preliminary experiments were performed by using a mixture of 600 μL of each selected dispersing solvent mixed with 200 μL of $CHCl_3$. The results, illustrated in Fig. 1B, indicated that acetonitrile exhibited the highest extraction efficiency followed by Me_2CO, whereas MeOH was not able to extract the analyte from honey solution. On the basis of these results, MeCN has been used as dispersant solvent in the extraction process.

After the selection of extraction/dispersive mixture, the effect of the pH of sample was investigated. The pH of samples solution could be a key factor, in liquid-liquid extraction as UA-DLLME, especially for the extraction of ionisable compounds. As know pH of aqueous solution could strongly influence the acid-base equilibrium of ionisable compounds and consequently the extraction efficiency (Campone et al. 2012). Therefore, in order to select the better pH of honey solution that promote the transfer of analyte in extraction solvent, the pH of honey solutions were adjusted at pH values ranged from 2 to 12, by little addition of 0,1 M of NaOH and HCl. The results showed that pH

within the range of 2–12 did not influence significantly ($p > 0.05$) the extraction efficiency of CAP, so the pH of the honey solution was not modified before performing the UA-DLLME.
Furthermore, the influence of ionic strength on the extraction efficiency of UA-DLLME, was studied. The salting-out effect has been universally used to influence the DLLME and LLE (Nagaraju & Huang 2007). Generally, addition of salt in the aqueous solution decreases the solubility of analytes and enhances their partitioning into the organic phase (LLE). In order to evaluate the effect of ionic strength on extraction efficiency, honey was diluted with a 5 mL of aqueous solution with NaCl 10% (*w*/*v*). As shown the Fig. 1S, when NaCl 10% was used to dissolve honey, the extraction efficiency of CAP strongly increased, because as expected, the solubility of analyte in aqueous phase decreases. On the basis of this result the use of NaCl as salting out agent was selected and its concentration as well as the volume of extraction and dispersant solvents were further optimized by the experimental design.

3.1.2. Optimization of UA-DLLME by experimental design

Once selected the main parameters that influence the extraction efficiency, the best amount of each parameter, which can affect the performance of UA-DLLME was carried out by using an experimental design (DOE) approach. The influence of three independent variables (NaCl %, volume of extraction and dispersive solvent) on extraction efficiency and matrix effects were simultaneously evaluating by a Box–Behnken design. Chromatographic peak area (PA) was considered as response variable to be maximized, whereas the matrix effect as variable to minimize, taking into account that the co-extracted may interfere (signal suppression or enhancement) the ionization of CAP. The experimental results obtained after UA-DLLME of PA and ME are shown in Table 1S. The coefficients of the quadratic models and the statistical significance of the regression coefficients were calculated by the response surface methodology. Standardised effect of each independent variable considered in DOE and their first order interactions were calculated by the analysis of variance (ANOVA) and the result are summarized in the pareto chart (Fig. 2S). As can be seen in Fig. 2S all considered factors and also their interaction (AC; AB; CC) exceed the blue vertical bar, which determines the statistical significance ($p < 0.05$) on CAP peak area. The combined effects of independent variables and the quadratic equations obtained excluding the non-significant factors ($P > 0.05$) was shown in the surface plots for PA (Fig. 3S). These quadratic models described the variability of PA at 99%. Regarding ME, after the exclusion of non-significant independent variable the values could not be adjusted to an appropriate model ($R^2 = 22\%$).This result could be probably explained by the low variability of response under different conditions (Table 1S), therefore matrix effect, was not considered in the selection of the optimized extraction conditions.
Finally, in order to maximize the extraction of the CAP and to select the best extraction condition a multiple response analysis was carried out. The optimized extraction condition extrapolated from the experiential design and used in the validation study were as follow volume of extraction solvent CH_3Cl 300 μL, volume of dispersive solvent CAN 800 μL and concentration of NaCl 15%.

3.2. Optimization of LC and MS/MS parameters

According to the decision No 657/2002/EC Commission Decision 2002/657/EC 2002 the confirmatory method used for the analysis of unauthorized substances as CAP listed in Group A of Annex I of Directive 96/23/EC shall provide information on its chemical structure with a minimum of 4 identification points (IPs). For this reason methods based on chromatographic separation coupled with the spectroscopic detection method are demanded. The IPs obtained in a specific analysis are listed in 657/2002/EC and depends on the technique used, a combination of liquid chromatographic separation coupled with low resolution mass spectrometer MS/MS, selecting 2 parent ions with 1 daughter for each parent, provided 5.0 IP, therefore even more than required by relation EC. The optimization of ESI source parameters was carried out by the infusion of a standard solution (MeOH/H_2O 1:1 *v*/v) at concentration of 5 μg mL^{-1}. The results shown that the analyte response obtained in the negative ionization mode was much high than signal obtained in positive mode, so

the negative ionization mode was used in the next acquisitions. Since CAP has the presence of two chlorine atoms in the molecule, the ESI (−) MS/MS of CAP spectrum showed the typical isotopic pattern with ^{35}Cl (most abundant with two ^{35}Cl) and ^{37}Cl (about 1/3 lower). So in order to obtain greater selectivity and sensitivity of the tandem mass spectrometer method we decide to use the two most intense daughter ions 256.9 of 320.9 and use the most abundant daughter ion 256.9 of 322.7. The most intense ion 320.9 > 256.9 has been choice as quantitative ion, while the equivalent fragments from ^{37}Cl molecule (322.7 > 256.9) was choose as confirmatory ions. In this way, selecting 2 precursor ions, each with 1 daughter the method achieves 5 identification points (Commission, E 2002) even higher than 4 identification points mandatory for the analysis of CAP residue in food. During the acquisition of samples in order to prevent the contamination of ESI source from matrix interferents, a divert valve was used to direct the HPLC eluate to ESI source only in the retention time windows of analyte (2-4 min.) whereas the other parts of chromatographic run were lead to the waste. This approach made possible to analyse a high number of samples without any decrease of signal response, thus avoiding to clean the mass spectrometer source. After the optimization of the MRM transitions different columns, solvents and flow rates were tested in order to choose the better chromatographic conditions for CAP. Regarding the selection of the analytical columns, since there was no need to separate complex mixtures of analytes, a short column, Kinetex C18 (50 × 2.1 mm) with small particle size (1.7 μm), was preferred due to shorter total run time and good peak shape.
Subsequently, the comparison of several mobile phase (H_2O, MeOH and ACN) and different solvent buffer (0.1% formic acid, 2 mM ammonium formate and their combination) were investigated. If on one hand, the difference in the ionization of analyte was not significantly influenced by the organic solvent, on the other hand the buffer composition in the mobile phase strongly affected the analyte ionization response. The addition of any tested buffer into mobile phase, strongly reduce the ionization of the analyte response (Fig. 2). On the basis of these results MeOH was chosen for its lower price than MeCN and no buffer was added. Finally, three different flow rates (0.3, 0.5, 0.7 mL min^{-1}) were tested in the gradient elution mode. Considering the ionization response and the backpressure of system, the flow rate of 0.3 mL min^{-1} was chosen. In these conditions, CAP elute at a retention time of 3.05 with the RSD% never exceeding 4.1%. Under the optimized condition the total LC-MS/MS run time per sample was 11 min (including column wash).

3.3. Method validation

3.3.1. Validation of UA-DLLME–GC–MS method and matrix effect evaluation

The proposed analytical procedure was validated in terms of selectivity, linearity, sensitivity, recovery, accuracy and precision, according to the European Commission Decision 657/2002 Commission Decision 2002/657/EC 2002. The UHPLC–MS/MS chromatogram of contaminate samples (A) and non-contaminated real sample (B) were showed in Fig. 3.
Data acquisition was carried out in MRM mode with two daughter ion of ^{35}Cl-CAP the most abundant I1 (320.9 > 256.9) was selected as quantify ion and one daughter ion of ^{37}Cl-CAP, I2 (322.7 > 256.9) as qualify ion; moreover, the MRM intensities ratio of (I1/I2) was used as additional identification criteria with a tolerance <10% of the expected ratio. In this manner, the developed method satisfied the EU guidelines for the confirmation of analytes with LC–MS/MS detection Commission Decision 2002/657/EC 2002.
In order to evaluate the selectivity of the method, ten CAP-free honey samples were analyses and no interfering peaks from the sample matrix were observed at the retention time of the CAP. The non-contaminate samples were also used to calculate standard deviation of noise of the noise amplitude of blank samples (σN) and the intercept of calibration curve was to force to pass through the σN. Calibration curves were constructed by spiking honey samples at different concentrations in the range from 0.1 to 5 ng mL^{-1}. The solvent calibration curve and matrix-matched curve has good linearity with a correlation coefficient (R^2) of 0.999 and 0.998 respectively (Fig. 4S).

Recoveries were determined by processing honey samples spiked at three CAP levels (0.1, 0.3 and 0.5 ng g^{-1}) each level was analysed in triplicate and recoveries found to be in the range of 54–60% (Table 1). Intra-day repeatability was expressed as the %RSD of the method after analysing five replicates of the analytes added at a concentration of 0.05 μg Kg^{-1} in honey were found to be <5%. The requirements of decision 657/2002/EC for the identification CAP in food of animal origin in introduce instead of the more often used method limit of detection (MLOD) and method limit of quantification (MLOQ) a new parameters called decision limit (CCα) and detection capability (CCβ).The practical meaning of these two new parameters can be resumed as follow: a signal ≤CCα will be declared compliant because the signal is a background noise, while a signal ≥CCβ will be declared non-compliant because the signal is produced from the forbidden substance.
Following the Eqs. (1), (2) the decision limit CCα and detection capability CCβ has been calculated for quantification ion and were 0.0115 and 0.0364 ng g^{-1} respectively (Table 1), these results are lower than limit imposed by EC (Commission Regulation EC 2009). In order to verify the absence of carryover phenomena, subsequently to an analysis of contaminate samples, a spiked honey at high concentration (2 ng g^{-1}) was analysed using developed method. After 10 injections of this spiked sample, an inter blank run (solvent MEOH: H_2O 1:9) has been injected and the absence of any chromatographic peaks in the retention time of CAP confirm no carry over phenomena. When mass spectrometry is use to quantify the analyte in complex matrices as foods, the ionization of analyte could be seriously affected by suppression or enhancement due to co-elution of interfering compounds, influenced negatively the accuracy and precision of method (Matuszewski, Constanzer, & Chavez-Eng 2003; Trufelli, Palma, Famiglini, & Cappiello 2011). Therefore, in order to select the suitable quantification method matrix effect (ME) was studied. Matrix effect was determined by comparing matrix-matched curves of pooled honey solution with standard solvent curve at same concentration (0.1–5 ng mL^{-1}) range. The results show that matrix-matched curve was comparable with the solvent curve (Fig. 4S) highlighting the total absence of matrix effects. Consequently, due to the response of CAP in honey was not conditioned by matrix effects, its quantification can be carried out using solvent calibration curve. The absence of matrix effect allows the method to avoid any other quantification strategy such as the use of internal standards, or matrix matched calibration, all procedures that increase the cost and time of the analysis.
After the validation study, in order to verify the practical applicability of the developed method, the analysis of CAP in 66 honey samples were carried out. Honeys of different botanical origin collected from beekeepers or from local markets in Campania region (38 samples), and 28 imported honeys (no information on the origin country) The overall results revealed that the contamination occurred among 5 (7.6%) samples, among these samples no sample collected from Italian market were contained CAP whereas 5 imported samples were contaminated with CAP at the concentration range of 0.038 to 1,62 ng g^{-1}. The other 61 samples (92,4%) did not show any CAP residue above CCβ. In agreement with literature data, the imported samples have CAP concentration higher than the local samples. This result further highlights the necessity to increase the analysed samples of imported samples in order to guarantee the highest quality and safety for the consumers.

4. Conclusions

An easy method for fast and reliable analysis of chloramphenicol in honey has been developed. The optimization of main parameters of extraction method, through an experimental design allow the determination chloramphenicol in honey sample at trace level. The quantification of CAP using tandem mass spectrometry with 2 specific MRM transitions of analyte enable selective and confirmatory detection allowing to achieve 5 identification points. The absence of matrix effect phenomena allow to quantify CAP with good precision and accuracy even at trace level avoiding the use of stable isotope-labeled CAP as internal standard. The developed method has been validated according to European Commission Decision 657/2002 criteria for the analysis of veterinary drug residues in food and successfully applied to 66 real samples. The quickness and easiness of the extraction technique combined with the high sensitivity and accuracy of the detection method are the

main advantages of proposed procedure. These features allow to increase the number of samples reducing at the same time solvents and other chemicals, making the method both environmentally friendly and inexpensive.

Conflicts of interest

The authors declare no conflict of interest.

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Table 1. Analytical performance of UA-DLLME method.

Analytical parameters	Values (RSD%)
CCa (µg kg^{-1})	0.0115
CCβ (µg kg^{-1})	0.0364
Recovery (0.1 µg kg^{-1})	54 ± (4)
Recovery (0.3 µg kg^{-1})	60 ± (5)
Recovery (0.5 µg kg^{-1})	52 ± (3)
Solvent curve	$R^2 = 0.999$
Matrix curve	$R^2 = 0.998$

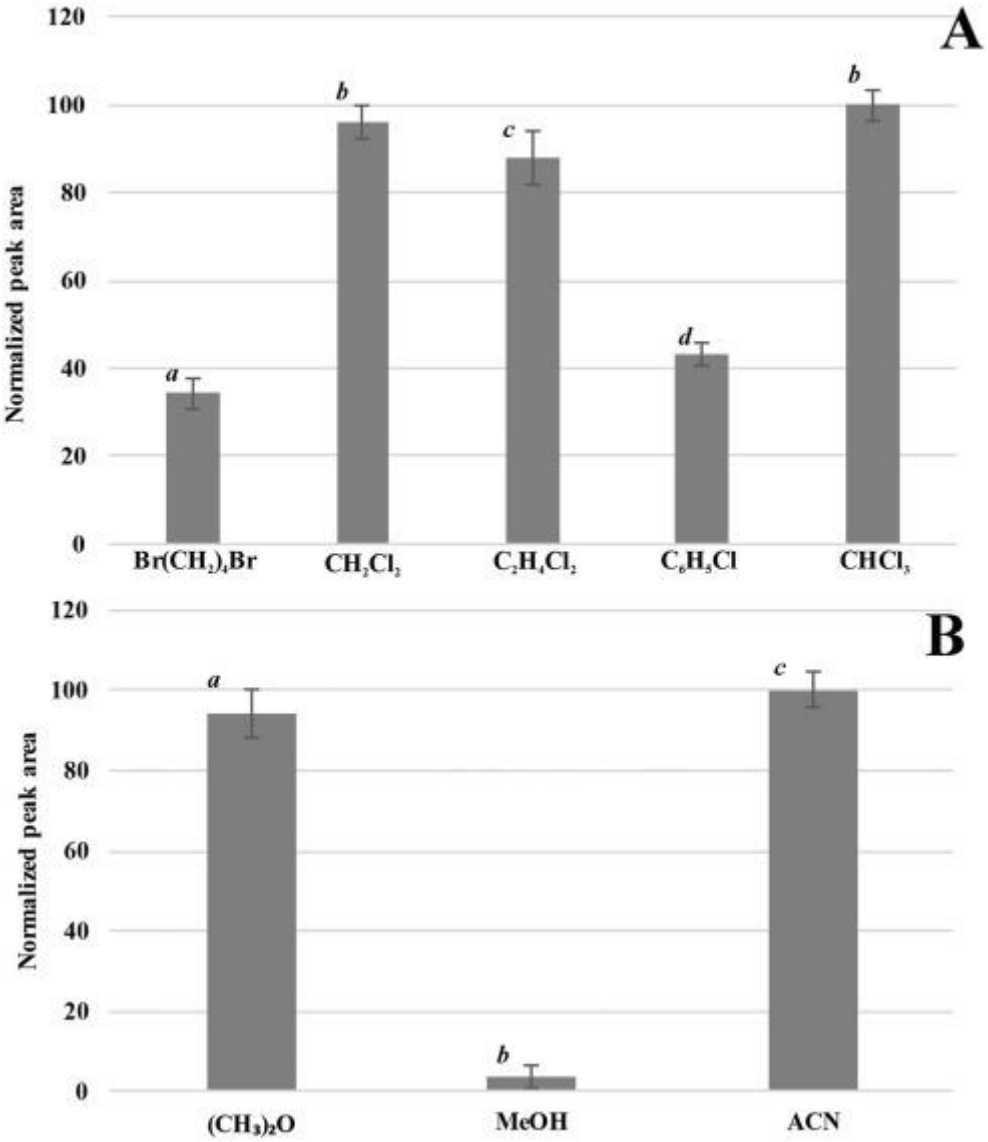

Fig. 1. Effect on extraction efficiency (expressed as normalized peak area) of UA-DLLME process under different extraction solvents (A) and dispersive solvents (B).

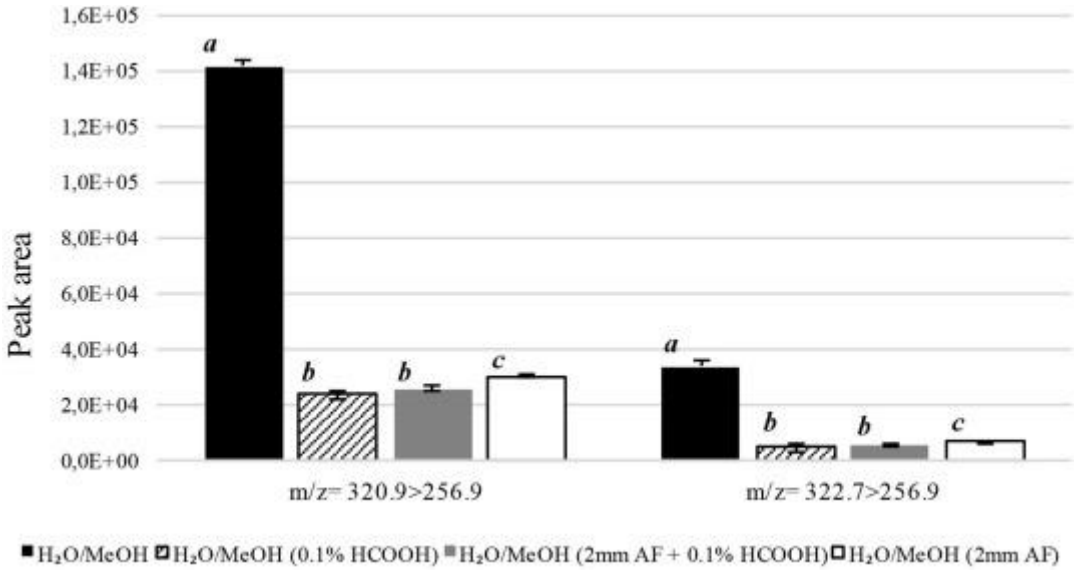

Fig. 2. Effect of different buffer composition of UHPLC/-MS/MS solvents on the ionization of the two selected MRM transitions of CAP.

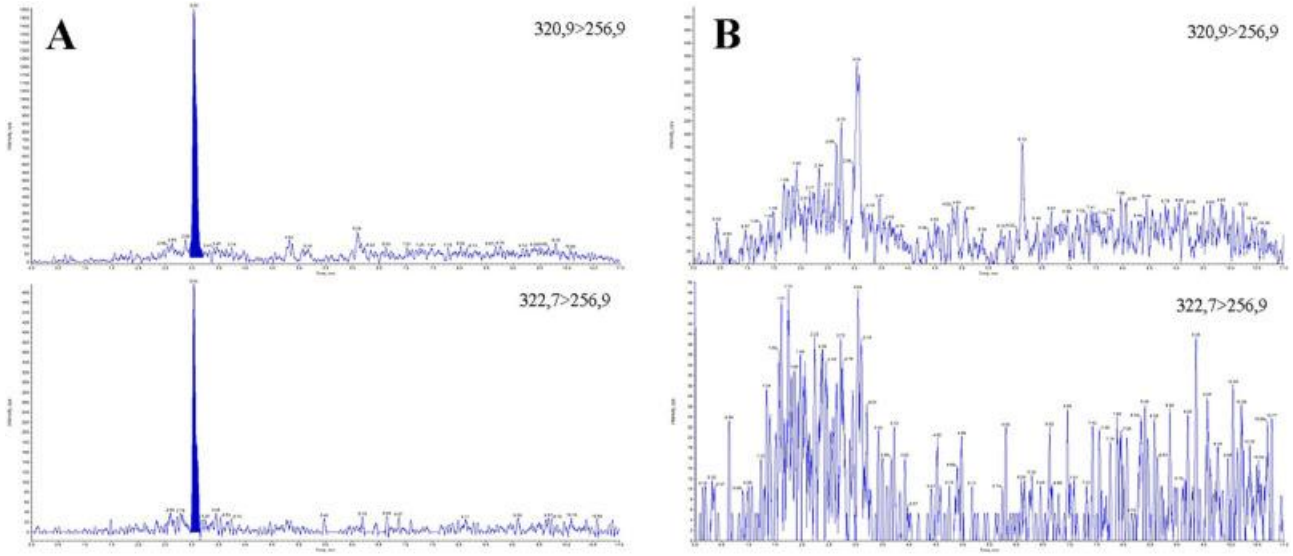

Fig. 3. Multiple reaction monitoring chromatograms of UA-DLLME of contaminated honey (A) and blank honey samples (B).

Supplementary Material

Figure 1S

Effect of salting out agent on extraction efficiency (expressed as normalized peak area) of UA-DLLME

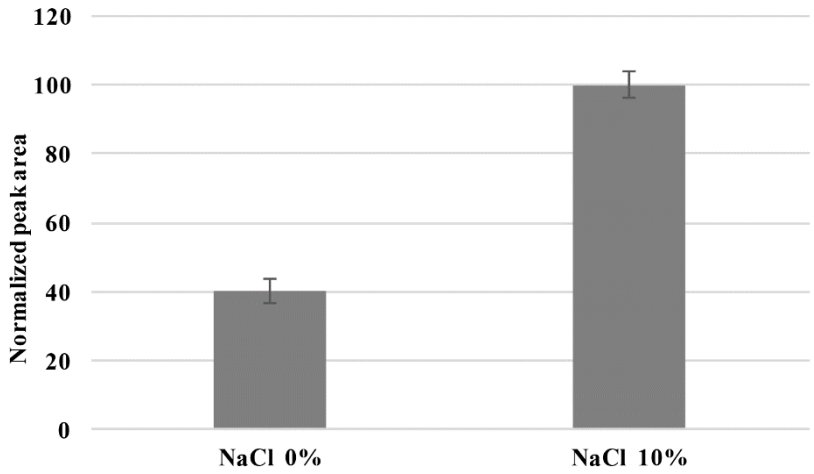

Figure 2S

Pareto chart of standardised effects for CAP response (peak area). The vertical line in the chart defines the 95% confidence level.

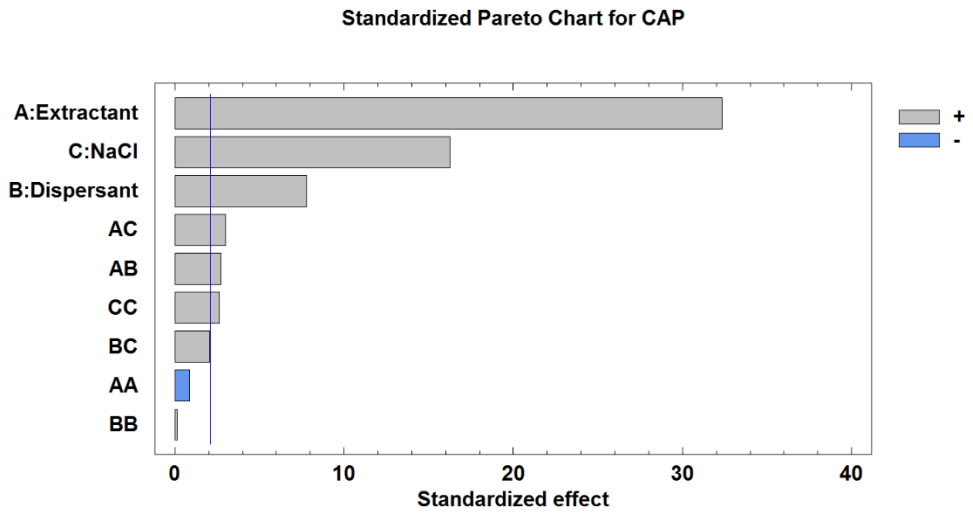

Figure 3S

UA-DLLME response surface plots of PA for the extraction of CAP

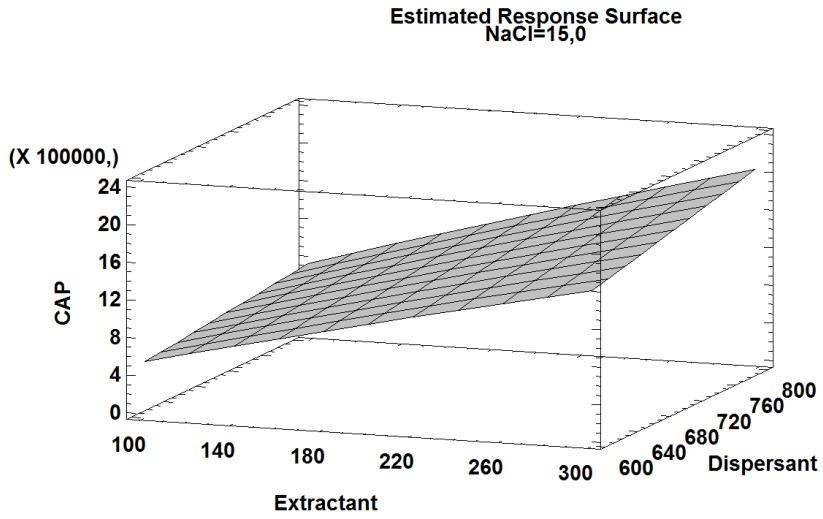

Figure 4S

Comparison of chloramphenicol peak area response both in solvent (MeOH 30 %) and non-contaminate pooled honey spiked in the range from 0.1 to 5 ng mL−1 under optimized experimental conditions.

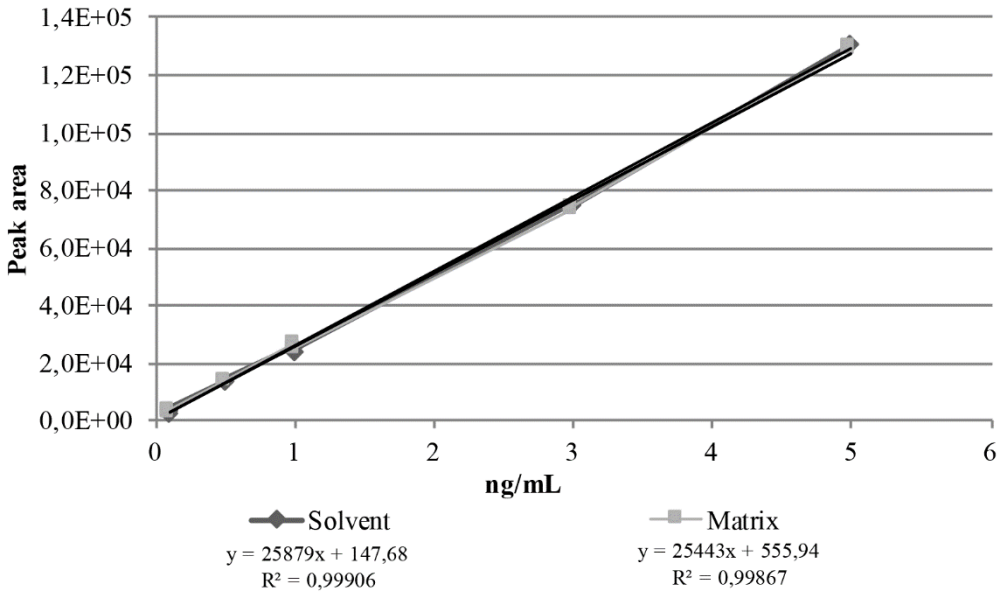

Table 1S

Experimental conditions of the response surface design (Box-Behnken design 2-factor interactions) and experimental values of the response variables: peaks area (PA) and matrix effects (ME)

Independent Variables				Response	
Block	**Extractant**	**Dispersant**	**NaCl**	**CAP**	**ME**
	µL	µL	%	(PA)	(%)
1	200	700	10	909858	-4
1	200	800	5	735775	-5
1	200	800	15	1472581	-3
1	300	700	15	1779950	7
1	100	700	5	212283	12
1	300	700	5	1140945	9
1	200	600	15	1095758	-4
1	100	800	10	406407	8
1	200	700	10	880009	7
1	100	600	10	257032	14
1	200	700	10	764233	18
1	100	700	15	549949	16

1	300	600	10	1246369	14
1	300	800	10	1566052	12
1	200	600	5	605994	13
2	200	700	10	931284	-2
2	200	800	5	806061	-3
2	200	800	15	1393176	-1
2	300	700	15	1762388	9
2	100	700	5	364959	14
2	300	700	5	1176540	12
2	200	600	15	1118059	-2
2	100	800	10	470222	11
2	200	700	10	948684	5
2	100	600	10	328134	12
2	200	700	10	1090605	15
2	100	700	15	711118	11
2	300	600	10	1242971	12
2	300	800	10	1709669	10
2	200	600	5	659608	11