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# Assessing the potentialities of an easy-to-use sample treatment strategy: Multivariate investigation on "Moka extraction" of typical ingredients from dietary supplements

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ABSTRACT

Mr. Bialetti invented Moka in 1933 and it still represents the most common way to prepare coffee at home. The process through which Mokas extract components from the ground coffee is a solid-liquid extraction which occurs at high pressure and temperature. These features are desirable in simple sample treatment strategies, since they allow good extraction efficiencies in a short time. Herein, for the first time, Moka-pot extraction was considered as an alternative processing protocol to extract polar compounds from dietary supplements. The effect of four experimental variables on extraction efficiency was evaluated through a fractional factorial design of experiments applied to a pooled matrix. In particular, solvent pH and its content of organic modifier, heating temperature and sample mass (reflecting the ratio to the amount of solvent which has to be kept fixed due to practical needs) were considered. The performances of the best conditions were then validated by determining recoveries (between 52 and 134 %, except for acetylsalicylic acid) and matrix effects (resulting always negligible or moderate at 100-fold dilution) of a spiked matrix which did not present any of the target analytes. They were finally applied to real samples, allowing to quantify some compounds, including artificial sweeteners, methylxanthines and taurine. Results were then compared with the quantities declared on the labels and those obtained with a Salt-Assisted Liquid-Liquid Extraction (SALLE), previously developed. Interestingly, the two methods were comparable for most compounds, but Moka extraction allowed to quantify taurine, which was not recovered with the SALLE. This promising result encourages further work to extend the use of the simple Moka device to other analytes and further matrices.

## 1. Introduction

Moka was invented in 1933 by Mr. Bialetti, who patented his product in 1950 [1]. In few years, Moka has diffused worldwide as one of the most common brewing methods to prepare coffee and to date hundreds of millions of Mokas have been sold [2]. Moka's success is due to its cheapness and simplicity: it consists of three main aluminum pieces: the boiling chamber containing the extracting water, the funnel filter in which the ground coffee is put, and the collecting chamber above. The latter two pieces are divided by a metallic filter (which guarantees that the coarser solids do not reach the collecting chamber) and a rubber gasket (that secures the sealing) [1,3]. Moka's peculiar configuration allows to extract the powdered coffee with a slightly higher pressure, temperature and speed, achieving good extraction efficiencies for caffeine and other flavoring and aromatic substances, generally associated with coffee's quality and its characteristics. Among the various types of coffee machines, Moka is the simplest and the cheapest, but even that one with the highest extracting yield [4,5]. This is assumed to be related with the double mechanism of extraction: after the *normal* phase in which the water is pulled up through the grounded coffee by uniformly filling the funnel filter, it occurs the so-called *volcanic* phase, in which the steam itself passes through the funnel allowing a vapor-liquid-solid extraction [2,3]. During the preparation of coffee beverages, this phase is generally undesirable, and thus avoided, since it is able to extract components generally associated with harsh bitter tastes of 'burnt' which lower the quality of the obtained coffee [2,4]. On the other hand, the whole process involved in the use of Moka (normal + volcanic phases), may be exploited for analytical extraction purposes.

Another source of caffeine is represented by Dietary Supplements (DS) for athletes, which can also contain other stimulant compounds and

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licit ingredients. Still, DS could be also faked or contaminated with doping compounds [6–8], defined as substances able to enhance the athlete's sport performances; for this reason most of the competitions are under the World Anti-Doping Agency control and legislation [9,10]. Among the methodologies employed in the processing of doping-related samples, there are common liquid-liquid extractions, alkaline extractions [11], solid-phase extractions, enzymatic hydrolyses [12–14], Quick Easy Cheap Effective Rugged and Safe (QuEChERS) extraction [15–17] and so on, including homogeneous liquid-liquid extractions, which allow a theoretical infinite contact surface area between sample and extraction solvent [18].

In this work, an analytical method for the extraction of polar compounds from DS using a Moka-Pot Extraction (MPE) was optimized by means of a fractional factorial design and validated. Another type of coffee machine has been already used for analytical purposes, but applied to different analytes and matrices: two 'hard-cap' machines were employed for the determination of polycyclic aromatic hydrocarbons in soils and sediments [19] and to extract veterinary pharmaceuticals from feedstuffs [20].

To the best of the authors' knowledge, this is the first analytical application of a MPE on matrices different from ground coffee [21]: along with the pressurized solid-liquid extraction occurring during the normal phase, the volcanic phase was exploited as well to ensure higher extraction efficiencies for the target compounds.

The analytes targeted in this study included licit compounds like methylxanthines and artificial sweeteners, as well as substances considered dopants, like diuretics, $\beta_2$-agonists and $\beta_2$-blockers. They are very polar and most of them present ionizable functions. Therefore, Hydrophilic Interaction LIquid Chromatography (HILIC) was employed, coupled with tandem Mass Spectrometry (MS) as detection system. The applicability of HILIC-MS/MS instruments to doping-related studies was already demonstrated [22,23], including the complementarity of this separation strategy with the more used Reversed Phase Liquid Chromatography (LC) [24].

Since no suitable certified reference materials were available, the performances of the definitive method were compared with another recently optimized procedure that involved a Salt-Assisted Liquid-Liquid Extraction (SALLE) applied to the same samples [25].

## 2. Materials and methods

### 2.1. Chemicals and reagents

LC-MS grade Acetonitrile (ACN), employed both as chromatographic eluent and as organic modifier of the Moka extracting solvent, was purchased from VWR (Fontenay-sous-Bois, France) as well as Methanol (MeOH) employed in the preparation of the stock-solutions, while ultrapure water was obtained in lab using a Milli-Q Millipore (Watford, UK) system. Formic acid (FA) and its ammonium salt ($FNH_4$) were employed as chromatographic additives and provided by VWR. NaOH from Fluka Analytical (Saint Gallen, Switzerland) was employed alternatively to acetic acid (AA, from VWR) as pH modifier of the Moka-extraction solvent. Moreover, washed and calcined quartz sand silica (Merck – Darmstadt, Germany) was employed as inert filler of the Moka's funnel filter.

Analytical standards (all above 98 % of purity) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA) acesulfame (ACS), furosemide (FRSM), hydrochlorothiazide (HCTZ), acetylsalicylic acid (ASA), paraxanthine (PRX), theobromine (TBR), theophylline (TFL), sucralose (SCL), taurine (TRN), cocaine (COCA), clenbuterol (CLBT), metoprolol (MTPL), terbutaline (TBTL), atenolol (ATN), and nicotine (NCT), with the exception of caffeine (CAFF) from Fluka Analytical and salbutamol (SLBT) from Alfa Aesar (Haverhill, MA, USA). A 2 μg $mL^{-1}$ mixture of standard solution was prepared in ACN:$H_2O$ 95:5 v/v from the single-compound stock solutions (at 109 - 4080 μg $mL^{-1}$ prepared in MeOH or MeOH:$H_2O$) and properly diluted to obtain daily working solutions.

### 2.2. Dietary supplements samples

Four different pre-workout DS, known to contain at least one of the target analytes, were bought online and aliquots of each DS were pooled to obtain a general matrix, to be used for subsequent Moka extraction optimization. The labelled compounds which were targeted were the four methylxanthines (CAFF, PRX, TFL, TBR), two artificial sweeteners (SCL and ACS), and taurine. Therefore, the optimization of the MPE involved the maximization of the extraction efficiency of these 7 analytes. Three DS were in a capsulated form and named C1, C2 and C3; while the fourth was a powder (thus named P1) supposed to be dissolved in water and drunk prior to and during the physical activity. Further information on these samples were given elsewhere [25].

A fifth DS, whose label declared vegetable components that should not be related to any of the target analytes, was used instead as a blank matrix to be spiked for the final recovery test, in which the method performances of every compound were assessed.

### 2.3. Moka pot extractions

#### 2.3.1. Design of experiments

When dealing with the optimization of a process, many variables could influence it contemporarily. Thus, it is advisable to employ a multivariate approach to understand their (linear, quadratic and interactive) effects, through the Design of Experiments (DoE) [26,27].

A wider discussion regarding the reasons of the specific DoE employed, as well as the experimental variables and their ranges, is reported in Section 3.1.1.

Briefly, a $2^{4-1}$ Fractional Factorial Design was applied, to limit the number of experiments to perform, obtaining some information about the interactions between variables [26]. Such a fractionation of a factorial design divides its experimental matrix in two symmetric multi-dimensional halves. The experimental variables selected as factors were: the temperature of the heating plate (125–175 °C) on which the Moka was placed; the amount of sample within the funnel filter (0.5–1.5 g - since these amounts were not enough to properly fill the filter, inert silica was employed as filler up to a total of 6 g within the funnel); presence of organic modifier (ACN) within the extraction solvent (0–15 %) and its pH (4–9, obtained by adding NaOH or AA). The fractionation half (plus the center point) selected as experimental plan, as well as the coded levels for each variable are summarized in Table 1.

The peak areas of the analytes, normalized for the actual amount of sample extracted in each experiment, were selected as responses and models (Multiple Linear Regression - MLR) were constructed by using the experimental plan as factors matrix. The exploratory Principal Components Analysis (PCA) on data, MLRs and predictions were all elaborated by using the Chemometric Agile Tool (CAT) software [28].

**Table 1**
Coded levels and experimental plan for the fractional factorial design employed in the optimization of the MPE.

| Factors | T [°C] | pH | Mass of sample [g] | % org.mod |
|---|---|---|---|---|
| Coded levels | | | | |
| **−1** | 125 | 4 | 0.5 | 0 |
| **0** | 150 | 6.5 | 1 | 7.5 |
| **+1** | 175 | 9 | 1.5 | 15 |
| Experimental Plan | | | | |
| **exp 1** | −1 | −1 | −1 | +1 |
| **exp 2** | +1 | +1 | −1 | +1 |
| **exp 3** | +1 | +1 | +1 | −1 |
| **exp 4** | 0 | 0 | 0 | 0 |
| **exp 5** | −1 | +1 | −1 | −1 |
| **exp 6** | +1 | −1 | −1 | −1 |
| **exp 7** | −1 | +1 | +1 | +1 |
| **exp 8** | +1 | −1 | +1 | +1 |
| **exp 9** | −1 | −1 | +1 | −1 |

#### 2.3.2. Moka-pot extraction procedure

Experiments were performed consecutively, after deep cleaning of the Moka in every inner and outer part. The first step in preparing Mokas was filling the boiling chamber with the proper amount of water, organic and pH modifier (when necessary). Secondly, the funnel filter was put onto the boiling chamber and the sample (DS previously mixed with inert silica) loaded in it. Then the collecting chamber was assembled and put onto the F60 heating plate (supplied by Falc Instruments, Treviglio, Italy), previously heated at the chosen temperature. After 10 min, during which the extraction occurs, Mokas were moved onto a trivet, allowing the extracts to reach room temperature prior to be transferred in a 50 mL volumetric flask, adjusting the volume by adding water. After that, a small amount of each extract was filtered on a 0.22 μm hydrophilic-PolyTetraFluoroEthylene filter (Thermo Scientific - San Jose, CA, USA) prior to being diluted in ACN:$H_2O$ 95:5 v/v and analyzed through HILIC-MS/MS.

The extracts contained very high amounts of CAFF, since they were all caffeine-based pre-workout DS. Hence, a high dilution factor (3-thousands times) was set to quantify it along with TRN and the sweeteners, while the remaining methylxanthines were quantified with a lower dilution factor (30 times). During the instrumental runs at lower dilution, the MS valve was switched in 'waste' from 3.7′ to 4.2′ of the run, to avoid massive injection of caffeine into the MS system.

#### 2.3.3. Validation of the optimal conditions

From the wider discussion in Section 3.1.2., the conditions identified as "potentially optimal" needed to be validated, by performing a one-way *t*-test to check if the experimental results (peak areas, normalized for the corresponding neat standard, observed from the extracts for each of the 7 analytes detected) were significantly different or not from the predicted values: confidence intervals were calculated and data were considered significantly different if those intervals were not overlapping [27].

The optimized conditions employed the lower amount of sample (0.5 g thus mixed with 5.5 g of inert silica) within the funnel filter, while the extraction solvent in the Moka's boiling chamber was set to contain 12.5 % of ACN (35 mL of water and 5 mL of ACN, without any pH modifier), with a temperature of the heating plate set at 170 °C.

Finally, due to the positive procedural validation of the processing method, two replicates of every single DS sample were processed with the selected conditions, aiming to detect and quantify the targeted analytes, by applying a dilution of 100- or 10.000- times.

### 2.4. HILIC-MS/MS analysis

The chromatographic separation was carried out on a YMC-Triart Diol-HILIC column (100 × 2.1 mm; 3 μm) by YMC Co. (Kyoto, Japan) installed on a 1200 series HPLC coupled to a 6430 triple quadrupole mass spectrometer by Agilent technologies (Santa Clara, CA, USA). This chromatograph was equipped with a binary pump, an online vacuum degasser, an automatic liquid sampler and a thermostated column compartment, while the MS/MS detector presented an ElectroSpray Ionization (ESI) ion source in a polarity switching mode. Operative conditions of the ESI source were set as follows: drying gas ($N_2$) at temperature and flow of 300 °C and 11 L $min^{-1}$ respectively, nebulizer pressure 15 psi, and capillary voltage 4000 V. Each target analyte was characterized through the Multiple Reaction Monitoring (MRM) mode, in which the precursor and product ions, as well as the fragmentor and collision energy of the 2nd quadrupole were optimized by using the Mass Hunter Optimizer software (version B.04.01) supplied by Agilent.

The HILIC separation employed in this work was previously developed for a subgroup of the targeted analytes [25]. Briefly, a combined flow and eluent gradient elution was employed with the mobile phases made of (A) $H_2O$ and (B) ACN:$H_2O$ (95:5, v/v), both containing 0.01 % FA and 0.2 mM $FNH_4$. The elution starts with a flow of 0.1 mL $min^{-1}$ and 100 % phase B, reaching up to 36.8 % of phase A and a flow of 0.3 mL $min^{-1}$ during the 25 min of analysis (restoring starting conditions time included). These chromatographic conditions allowed a satisfactory separation even for the compounds not included in that previous work [25] as reported by the retention times listed in the Supplementary Material (Table S.1).

The MRM conditions of the analytes not previously studied were optimized by following the same procedure to achieve the MRM parameters, i.e. by repeated injections in the MS system of 10 μL of single standard solutions at a concentration of 500 μg $L^{-1}$ in ACN:$H_2O$ (85:15, v/v). All these parameters, as well as some physico-chemical properties of the analytes, are summarized in Table S.1.

### 2.5. Method performances

The final procedure was assessed in terms of analytes process efficiency (recovery and matrix effect), Limits Of Detection (LOD) and Quantification (LOQ), specificity and precision.

Recoveries were assessed by processing 3 aliquots of a spiked matrix that did not naturally contain any of the analytes (checked by the parallel processing of a non-spiked aliquot) as described in Section 2.2. In detail, analytes were spiked at two different concentration levels, according to the expected amounts in real samples: 50 μg of ACS, CAFF, SCL and TRN and 5 μg of the remaining compounds were spiked onto the 0.5 g aliquots of the blank-DS, by using 350 μL of a concentrated mix of standards. Each of these aliquots was subjected to MPE as described in Section 2.3.2. and the filtrated extracts were furtherly diluted 10- or 100- times (according to the group of analytes of interest), in order to get a corresponding 10 μg $L^{-1}$ of concentration in the case of a 100 % recovery, which was evaluated as follow for each analyte [29]:

$$R\ (\%) = \frac{S_{SB} - S_{NS}}{S_{SA} - S_{NS}} \cdot 100\% \quad (1)$$

Where $S_{SB}$, $S_{SA}$ and $S_{NS}$ are the analytical signals (peak areas) of the three types of samples analyzed: the non spiked diluted aliquot (NS), the same aliquot, spiked with 10 μg $L^{-1}$ of each analyte after the processing (corresponding to the quantitative recovery of the analytes – SA) and the aliquots spiked before processing (SB).

A diluted aliquot of each extract was further spiked with 10 μg $L^{-1}$ of the analytes, allowing to determine also the matrix effect (ME), expressed as the signal alteration (suppression or enhancement) with respect to a neat standard ($S_{NEAT}$) of the same concentration (ME=100 % meaning no matrix effect), by applying the following equation [27]:

$$ME\ (\%) = \frac{S_{SA} - S_{NS}}{S_{NEAT}} \cdot 100\% \quad (2)$$

This was calculated for the real samples' extracts, too.

Instrumental LODs and LOQs were assessed as the concentrations corresponding to a signal presenting a signal-to-noise ratio of 3 and 10, respectively. For the corresponding in-matrix values, the dilution factors due to sample pre-processing, as well as the correction for procedural recoveries and matrix effect were applied. This strategy was employed since it resulted more conservative (in a previous work with the same kind of samples and instrumental analysis) than the ratio between the standard deviation of the matrix blanks' areas and the angular coefficient of the calibration curve [25,30].

Specificity was assessed by comparison of a reference standard for retention times of the analytes and, when possible, by calculating the ratios between the qualifier and quantifier transitions of each compound, monitoring that they maintain a deviation within $a \pm 30$ % from the standard itself, as recommended by some guidelines [31]. Intra-day instrumental precision was assessed during each day of analysis, by repeatedly injecting neat standards at three levels (0.2, 2 and 20 μg $L^{-1}$) and evaluating the Relative Standard Deviation (RSD %) of each analyte's peak areas. Inter-day instrumental precision was assessed in the same way, but calculating the RSD % of the peak areas of each analyte at those three levels on all the injections performed during three

non-consecutive days.

Moreover, this innovative strategy was compared to the previously employed SALLE [25] in terms of extraction efficiencies and greenness. The former was assessed through the comparison of the methods' performances and by executing two-way *t*-tests to establish if the quantification results of the two strategies were significantly different or not. Meanwhile, the latter aspect was assessed with the Analytical Greenness (AGREE) Metric Approach [32], by applying different weights (reported in Table S.2) based on their meaningfulness related to this work. Finally, the AGREE score was also calculated for an imaginary Moka 10-times smaller than that actually employed, predicting the effects of a potential miniaturization of MPE. However, such a Moka should be produced *ad hoc*, since there are no commercially available Mokas smaller than the used one.

## 3. Results and discussion

### 3.1. Method development

#### 3.1.1. Experimental design conditions

As briefly introduced in Section 2.3.1., a DoE approach was recommended in such a study. Therefore, the flowchart reported by Benedetti et al. [27] presenting the fundamental steps of a DoE was followed. Firstly, the aim of this study was to maximize the extraction efficiency of the 7 target analytes from DS with a Moka. This was monitored by taking as responses the peak areas obtained from the HILIC-MS/MS analyses of the diluted extracts, normalized for the amount of sample processed.

After that, the variables with a possible influence on the process and their respective experimental ranges have to be selected. It is known that several variables can modify the extraction process, including the contact time between the water and ground coffee, water temperature and pressure, the ground coffee/water ratio, the eventual volcanic phase and Moka's structural features [5]. The latest two were kept constant by allowing each extraction to be exhaustive (10 min on the heating plate were enough to this aim) and by using always the same (carefully washed) Moka. Both the contact time and the water temperature and pressure are somewhat dependent on the heating temperature which was selected in the range of 125–175 °C, while the coffee/water ratio was considered as the amount of sample to be extracted. In fact, Mokas work properly only if correctly filled and a certain, fixed, amount of boiling solvent is required (in this case, the smallest commercially available Moka, 40 mL). Still, the selected amount of sample was far too low to guarantee a uniform distribution within the Moka's funnel filter, and this would have led to a low reproducibility of the extraction efficiency. Therefore, inert silica was employed as filler, properly homogenized with the sample, to reach a total amount of 6 g inside the funnel. It is important to keep in mind that the actual extraction conditions (including pressure and other kinetics factors) depend on structural features of the specific Moka employed. Like many other analytical methodologies, the optimal conditions may be specific for the actual instrumentations and materials employed (in this work, the Moka pot).

When preparing coffee with Moka, the extraction solvent is simply water. But in an analytical method, its characteristics could be significant and then even solvent pH and percentage of organic modifier – ACN – within the extraction solvent were considered as experimental variables.

The next step involves the selection of the specific DoE. One of the easiest design is the full factorial, which requires to perform $2^k$ experiments (where $k$ is the number of variables involved, in this case, 4) allowing to estimate the variables' effects and their interactions [27]. Still, with the amount of pooled sample available, $2^4$=16 experiments were unaffordable. In fact, the selected samples were previously employed in the optimization of another extraction procedure [25].

A more accessible alternative is represented by the Plackett-Burman design [33], a screening design which requires to perform $4n > k$ experiments (where $n$ is an integer) without obtaining any information about the interactions [27], thus requiring 8 experiments, in this case. A better compromise was represented by the $2^{4-1}$ fractional factorial design, because with the same number of experiments as a Plackett-Burman DoE, it was able to provide some (but not exhaustive) information about the interaction among the variables. In such a design, the intercept is "confounded" with the quaternary interaction (very unlikely), the main effects are confounded with the ternary interactions (quite unlikely), and couple interactions are confounded among each other (more probable) [26]. From a mathematical point of view, further experiments would be required to discern the confounded contributions of the factors that resulted significant.

Experiments were therefore performed according to the experimental plan reported in Table 1 and data were elaborated as discussed in the next section.

#### 3.1.2. Data elaboration: determination of the optimal conditions

The data matrix obtained by performing the experiments was built using the experimental plan reported in Table 1 as factors, and the normalized peak areas of the 7 analytes as responses. Thus, a regression model could be computed for each response. Nevertheless, a global overview of the results can be achieved by performing a PCA on autoscaled data (responses) with CAT, which highlighted that most of the experimental variance was explained by the first PCs (77.4 % and 15.5 % for PC1 and PC2, respectively).

As graphically described by Fig. 1, the analytes heterogeneously distributed along PC2, while they presented similar and positive loadings on PC1, which explained a significant percentage of the variance. This suggested that higher scores on PC1 maximized extraction efficiencies. Hence, as a first approach a regression model was computed using the PC1 scores as unique response.

Since the fractional factorial DoE used implied that some of the effects were "confounded" [26], from a chemometric point of view further experiments would have been required to discriminate the confounded effects. From an analytical perspective and knowing the problem, it could be assumed that three-terms (or even four-terms) interactions are expected to be not significant. Thus, the first four coefficients of the obtained model (coefficients plot reported in Fig. 2A) can be reasonably assigned to the single variables. By looking at Fig. 2A, it can be deduced that the most relevant variable is the mass of sample, with a negative

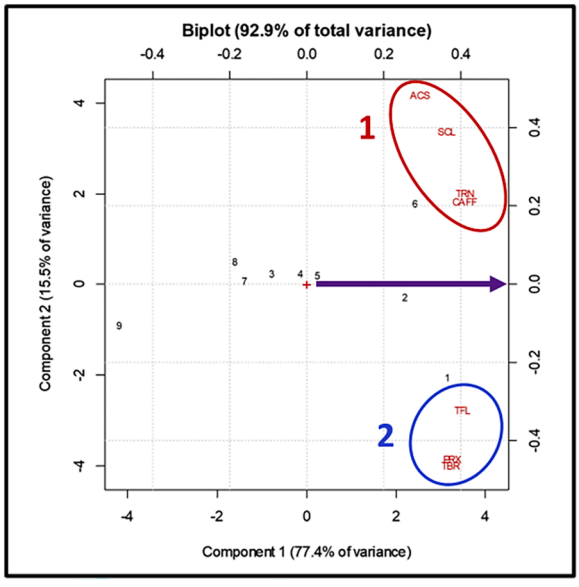


**Fig. 1.** Biplot of the PCA performed on the analytes' peak areas obtained in the 9 experiments of the fractional factorial design. Analytes are distributed along PC2 (forming 2 groups), but they present similar loadings on PC1. For acronyms, refer to paragraph 2.1.

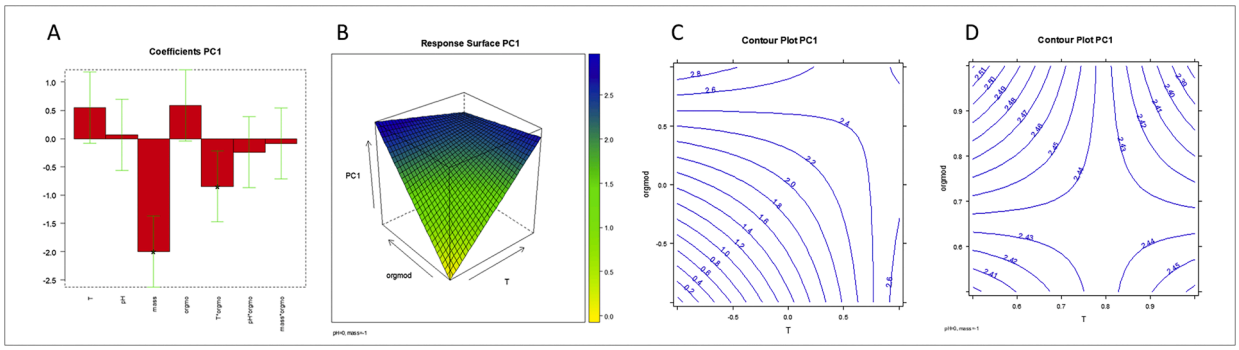


**Fig. 2.** Regression model on PC1. A) coefficients plot of the model built on PC1; B) response surface and C) contour plot at fixed levels (pH=0, mass=−1); D) zoom of the contour plot on the inflection point (coded levels: $T$ = 0.79, org.mod.=0.65) region.

coefficient. Moreover, both heating temperature and acetonitrile percentage presented a positive effect in increasing the extraction efficiency; nevertheless, their coefficients resulted not significant due to the high uncertainty intervals (due to the limited degrees of freedom). Finally, solvent pH presented a very small coefficient, resulting non-significant.

The interpretation of the interaction coefficients is less straightforward. In fact, their effects were confounded by the model in couples, and generally two-terms interactions are quite frequent [26]. One interaction coefficient resulted significant with a small negative effect, but mathematically it was not possible to discern the contributions of the two aliased interactions (temperature – organic modifier and solvent pH – mass of sample interactions). Still, it is unlikely that the amount of sample could influence the effect of solvent pH in extraction efficiency and vice versa, while it is much more realistic that the heating temperature could interact with the amount of organic modifier present in the boiling chamber. Therefore, the latter interaction was considered to be the significant one. Their combined effect on PC1 scores is shown in Fig. 2B and C, that report the response surface and the contour plot of the model by fixing mass of sample (−1) and solvent pH (0) levels. The contour plot highlights the presence of an inflection point (zoomed in Fig. 2D), which could represent the best compromise for the two groups of compounds.

In fact, temperature and organic modifier presented divergent effects for the two groups of analytes (referred to the ellipses in Fig. 1): peak areas of ACS, SCL, CAFF and TRN (group 1) resulted maximized at high temperature and low organic modifier, while TBR, PRX and TFL (group 2) at low temperature and high organic modifier, as shown in Fig. 3 by the response surfaces of CAFF (as an example of group 1 analytes, on the left square) and TFL (group 2, right one).

From these response surfaces, it became evident that maximizing PC1 would not be a good choice, because these variables influenced PC2 as well, which discriminated the two groups of analytes. Since all of them have to be maximally recovered, it was necessary to find the best compromise, which could be represented by the inflection point of the response surface of PC1 highlighted in Fig. 2D. The coded levels of the inflection point operatively correspond to $T$ = 170 °C and org. mod.=12.5 % (5 out of the 40 mL), and these MPE conditions were firstly applied to the pooled sample to validate the models, secondly to the spiked blank DS to assess the method performances, and consequently to the single real samples for quantification purposes.

As anticipated in Section 2.3.3., the results experimentally obtained by the MPE of the pooled samples through the conditions suggested by the inflection point were compared with those predicted by the models at the same values. The one-way *t*-tests showed that experimental values were always not significantly different (95 %) from the predicted ones, with the exception of TRN, for which resulted about five times lower. This difference could be ascribed to many factors, including chemometric aspects like the presence of quadratic terms that were ignored during the construction of the model, or even to a non-heterogeneous distribution of TRN within the pool. Nevertheless, the inflection point conditions were considered satisfactory. Thus, they were applied to spiked and non-spiked aliquots of the "blank"-DS to assess the method performances, as described in Section 2.5.

### 3.2. Method performances

Method performances were assessed as described in Section 2.5 and they are herein presented, being summarized in Table 2. Moreover, an extensive comparison (for the analytes in common) with those of the

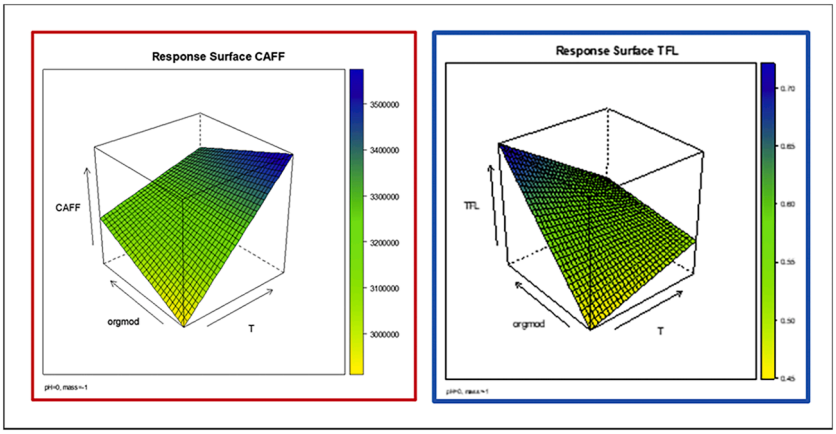


**Fig. 3.** Surface responses of case studies for group 1 (caffeine. CAFF, squared in red) and 2 (theophylline - TFL, in blue), obtained at the fixed coded levels of pH (0) and mass of sample (−1).

**Table 2**
Method performances of the optimized MPE, including analytes recoveries (R%) and matrix effects (ME %), intra-day precision at three level and procedural limits of detection and quantification.

| Analyte | Extraction efficiency | | Intra-day Precision RSD% ($n \geq 8$)[a] | | | Procedural sensitivity | |
|---|---|---|---|---|---|---|---|
| | R (%) ($n = 3$) | ME (%) ($n = 3$) | 0.2 μg $L^{-1}$ | 2 μg $L^{-1}$ | 20 μg $L^{-1}$ | LOD (μg $g^{-1}$) | LOQ (μg $g^{-1}$) |
| ACS | 96 ± 9 % | 91 % | 6 % | 5 % | 3 % | 0.09 | 0.28 |
| FRSM | 71 ± 6 % | 109 % | 13 % | 4 % | 4 % | 0.3 | 0.8 |
| ASA | 18 ± 3 %[a] | 94 % | 6 % | 9 % | 7 % | 1 | 4 |
| HCTZ | 62 ± 9 % | 102 % | 8 % | 7 % | 5 % | 0.3 | 1 |
| CAFF | 85 ± 14 % | 103 % | 5 % | 4 % | 4 % | 1 | 3 |
| TFL | 87 ± 5 % | 106 % | 6 % | 6 % | 6 % | 0.3 | 1 |
| SCL | 77 ± 13 % | 100 % | 23 % | 9 % | 9 % | 0.5 | 1.6 |
| TBR | 98 ± 8 % | 124 % | 15 % | 8 % | 6 % | 0.2 | 0.5 |
| PRX | 93 ± 12 % | 120 % | 12 % | 6 % | 6 % | 0.2 | 0.6 |
| TRN | 57 ± 2 % | 117 % | 13 % | 4 % | 4 % | 1 | 3 |
| COCA | 52 ± 8 % | 123 % | 6 % | 6 % | 1 % | 0.03 | 0.1 |
| ATN | 74 ± 7 % | 152 % | 9 % | 8 % | 8 % | 0.2 | 0.6 |
| NCT | 63 ± 4 % | 102 % | 3 % | 8 % | 1 % | 1 | 5 |
| CLBT | 72 ± 9 % | 83 % | 4 % | 5 % | 7 % | 0.04 | 0.11 |
| MTPL | 84 ± 7 % | 103 % | 5 % | 14 % | 4 % | 0.1 | 0.3 |
| TBTL | 104 ± 13 % | 109 % | 8 % | 2 % | 3 % | 0.02 | 0.06 |
| SLBT | 134 ± 23 % | 78 % | 24 % | 7 % | 9 % | 0.3 | 0.9 |

[a] Fictitious recovery, due to the significant ASA hydrolysis during the sample processing [34].

previous work involving the same samples [25] was carried out.

The procedure on the blank DS allowed to determine the extraction efficiency for each of the targeted analytes. Recoveries (R, in the table) ranged from 52 to 134 %. The only exception was ASA (apparent recovery, 18 %) probably due to its degradation in the operative solutions [34]. MPE allowed very good recoveries, and generally comparable with those previously obtained by SALLE [25], with significant improvement for the recovery of ACS, SLBT and the dimethylxanthines (TFL, TBR and PRX) and without unacceptable decreases: the worst case was COCA, from 72 to 52 %. Matrix effects (ME) observed at such dilutions (100-fold or higher) were generally negligible, being occasionally moderate (overall ranging from 78 to 152 %). This is in total agreement with what already observed processing these samples through SALLE [25].

Procedural LODs and LOQs allow to detect even minor components within the DS, being in the ranges 0.02–1 μg $g^{-1}$ and 0.06–5 μg $g^{-1}$, respectively. These values are not easily comparable with those obtained by other works, since they are calculated at the actual dilution, which is enough to detect the declared compounds and that did not reveal any further analyte present. Still, lower dilution factors would allow lower LODs and LOQs (always checking if matrix effects are acceptable).

Specificity was guaranteed by checking the retention times and the ratio between the available MRM transitions: the qualifier/quantifier ratio was automatically calculated by the MassHunter Quantitative Software and no outliers were detected, being all within $a \pm 30$ % difference compared to the reference standard as recommended by some guidelines [31]. Following the EU directive, the RT and each MRM transition provided 1 and 1.5 identification points, respectively [35]. Thus, 5 points for each compound were achieved, with the exception of the three dimethylxanthines and taurine for which a single MRM transition was monitored (and thus 3.5 points were achieved). This guarantees high confidence in identity confirmation of the target analytes, since the guidelines recommend 3–4 identification points [35]. Instrumental intraday precision was particularly satisfying, since the RSD % of the peak areas resulted always lower than 15 %. The only exceptions were obtained for SCL and SLBT at the lower level, which is quite close to their instrumental LOD. On the contrary, instrumental inter-day precision presented very high values (Table S.3). This is due to the significant variability in the MS performances, which are quite known to be subjected to long-term fluctuations (analyses were performed during non consecutive days) [36]. This considered, it was necessary to analyze a complete set of calibration standards during each batch of analysis.

Regarding the greenness of this work, the MPE was optimized performing a limited number of experiments and employing a limited amount of sample (fundamental aspect, considering the low leftovers from the previous work [25]), thanks to the DoE strategy. The recent metric developed by Nowak et al. [37] indeed suggested that also the number of experiments in method development should be minimized, for higher sustainability. From an analytical point of view, the AGREE metrics [32] gave a lower score to this MPE compared to SALLE, as reported in Fig. 4. The slight differences were mainly due to the amount of waste generated and to the volume of the toxic solvent (ACN) employed per sample (principle 7 and 11, respectively). This aspect could be improved if a smaller Moka were available: hypothesizing a 10-times smaller Moka, an even higher score than that of SALLE would be achieved.

Fig. 4 clearly shows that the principles that worst influenced the AGREE scores were the third and the ninth, corresponding to the positioning of the analytical devices (off-situ) and their energy consumption. For different reasons, their weights were reduced: firstly, it is impossible to perform analysis in-situ in such a study (application on a product); secondly, the optimization was not related to the instrumental analysis (involving a high-energy consumption device like HILIC-MS/MS), that still allowed sensitivity and specificity otherwise almost impossible to achieve.

### 3.3. Quantification

All the targeted analytes were investigated in single DS extracts, but only the seven compounds for which the method was optimized were detected at least once. Comparing the results with those obtained by SALLE (correcting each analyte for its recovery) allowed interesting considerations (Table 3). Still, MPE resulted very efficient in the extraction of TRN compared to SALLE (about 3 order of magnitude higher quantification) in P1 extracts: 391 ± 32 mg $g^{-1}$ rather than few μg $g^{-1}$. It is worth noticing that TRN was not considered during the optimization of SALLE procedure [25], and from preliminary results, it appeared to have a very poor recovery with that processing method.

Table 3 clearly shows that MPE extraction quantifications were often comparable with those from SALLE for most of the analytes, resulting not significantly different in 59 % of the cases from the results of the two-ways *t*-tests performed. For most of the remaining quantifications, it presented a slightly higher result for ACS and lower for CAFF, TBR and PRX in some samples.

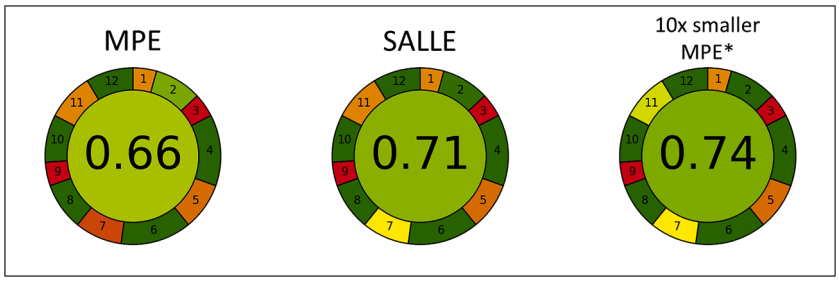


**Fig. 4.** AGREE Scores of the MPE procedure compared with that of SALLE from [23] and that of a hypothetical MPE performed with a Moka 10-times smaller (*- it remarks that this method is not available, at the moment) – The numbers reflect the Green Analytical Chemistry principles to which every score is related (reported in Table S2) [31].

**Table 3**
Amounts of analytes detected per gram of the DS samples (C1, C2, C3 and P1). MPE results (this work) derived from two independent measurements; data of SALLE methodology from [25].

| Analyte | Method | C1 | C2 | C3 | P1 |
|---|---|---|---|---|---|
| ACS [mg $g^{-1}$] | MPE[c] | ND | ND | ND | 13.3 ± 0.2 [a] |
| | SALLE | ND | ND | ND | 7.66 ± 0.04 |
| CAFF [mg $g^{-1}$] | MPE [c] | 50 ± 8 [b] | 283 ± 18 [b] | 137.9 ± 0.3 [b] | 27 ± 1 [a] |
| | SALLE | 51.9 ± 0.8 | 243.3 ± 0.6 | 134 ± 4 | 34 ± 1 |
| TFL [μg $g^{-1}$] | MPE [d] | 69 ± 10 [b] | 21.5 ± 0.8 [b] | 17.4 ± 0.1 [b] | ND |
| | SALLE | 70 ± 1 | 20 ± 3 | 18 ± 2 | ND |
| SCL [mg $g^{-1}$] | MPE [c] | ND | ND | ND | 8.6 ± 0.3 [b] |
| | SALLE | ND | ND | ND | 7.9 ± 0.3 |
| TBR [μg $g^{-1}$] | MPE [d] | 81 ± 15 [b] | 486 ± 12 [b] | 26.2 ± 0.1 [a] | ND |
| | SALLE | 111 ± 2 | 575 ± 25 | 45 ± 5 | ND |
| PRX [μg $g^{-1}$] | MPE [d] | 22 ± 4 [b] | 29.6 ± 0.6 [a] | 29.5 ± 0.1 [a] | 9.6 ± 0.7 [a] |
| | SALLE | 27.6 ± 0.4 | 38 ± 2 | 52 ± 1 | 19.6 ± 0.2 |

[a] MPE results significantly different (95 %) from those obtained by SALLE.
[b] MPE results not significantly different (95 %) from those obtained by SALLE.
[c] Quantification obtained from aliquots diluted 10.000- fold dilution factor.
[d] Quantification obtained from aliquots diluted 100- fold dilution factor.

The detected amounts of these targeted analytes were compared to those declared from the labels of the DS. The only analytes with a declared amount on the DS labels was CAFF (in each DS) and TRN (in P1), while the presence of the other methylxanthines was deduced from the plant extracts ingredients known to contain them and the artificial sweeteners were just labelled as "other ingredients" [25]. Regarding caffeine, it was already found that the declared amounts were reasonably reported in the labels (except for C2, which was unclear) [25] and, considering the servings indicated, they were close to the recommended maximum single-dose intake of 200 mg [38,39], except for P1 which exceeded that limit, considering the 9 g dose indicated on the label. Also taurine presented some issues in the same DS: it was found at a higher level than the indicated amount (1000 mg per 9 g serving, meaning approximately 111 mg per gram of product). Thus, the present results indicated that this DS exceeds taurine's limits in dietary supplements as well: it is above the maximum intake allowed in Italy (1000 mg) [40] and slightly above the Canadian one (3000 mg) [41]. Still, its intake should not represent any risk for human health, since a safe level of 6 g of taurine per day was estimated [42].

## 4. Conclusions

In this work, DoE allowed the development and optimization of an innovative and effective sample treatment strategy by employing a limited amount of sample. The method optimization included the execution of the experimental plan and the construction of the models, their validation and the following application of the selected conditions to real samples, in order to assess method performances and perform a quantitative analysis.

The optimized MPE was satisfactory in terms of specificity, sensitivity, repeatability (intra-day precision), quantification, and greenness. If compared with a previous method applied to the same samples [23], MPE allowed generally better recoveries.

These preliminary data suggest that MPE applied to DS is promising, thanks to its cost-effectiveness and ease of use. Additional studies could be addressed to reduce the operational quantities by building a smaller Moka. This could further increase the greenness of the procedure, as demonstrated by the higher AGREE score that a 10-times smaller Moka would allow to achieve. Furthermore, MPE could be extended to different classes of analytes and especially matrices to be processed.

## CRediT authorship contribution statement

**Matteo Baglietto:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Barbara Benedetti:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Marina Di Carro:** Writing – review & editing, Supervision, Project administration. **Emanuele Magi:** Writing – review & editing, Supervision, Project administration.

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2024.100110.

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