Innovative thermally assisted on-line solid phase extraction-reversed phase liquid chromatography applied to targeted nutrimetabolomics in human biofluids

Lapo Renai [a,1], Luca Marzullo [a,1], Giulia Bonaccorso [a], Serena Orlandini [a], Fulvio Mattivi [b], Maria Concetta Bruzzoniti [c], Massimo Del Bubba [a,*]

[a] *Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019, Sesto Fiorentino, Florence, Italy*
[b] *Metabolomics Unit, Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via Mach 1, 38098, San Michele all'Adige, Trento, Italy*
[c] *Department of Chemistry, University of Turin, Via Pietro Giuria 7, 10125, Turin, Italy*

HIGHLIGHTS

- A novel combination of thermal desorption with on-line SPE is proposed.
- Two different thermal devices have been implemented and investigated.
- Thermally assisted desorption (TD) of strongly retained analytes is demonstrated.
- TD on-line SPE coupled with reversed-phase liquid chromatography is allowed.
- This strategy succeeded in the analysis of gut metabolites in biofluids.

GRAPHICAL ABSTRACT
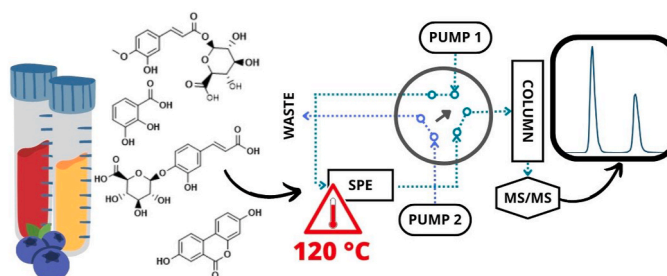
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ABSTRACT

In this study, the use of thermal desorption in on-line solid phase extraction coupled with reversed phase liquid chromatography (on-line SPE-LC) was for the first time proposed and demonstrated for the desorption of analytes strongly retained by multiple interaction polymeric sorbents. In detail, this analytical strategy was applied to the on-line SPE-LC targeted analysis of a model set of 34 human gut metabolites characterized by heterogeneous physicochemical properties (i.e., octanol–water partition coefficient in the range $-0.3 - 3.4$). The novel thermally assisted on-line SPE approach was investigated in comparison to conventional room temperature desorption strategies based on the use of (i) an optimized elution gradient or (ii) organic desorption followed by post-cartridge dilution. The thermally assisted desorption strategy has been shown to be better performing and suitable for the development of a reliable and sensitive method for the analysis of the model group of analytes in urine and serum. In more detail, under the optimized experimental conditions, the proposed method provided negligible matrix effects in both biofluids for almost all target analytes. Moreover, method quantification limits were in the ranges 0.026–7.2 µg L^{-1} and 0.033–23 µg L^{-1} for urine and serum, respectively, i.e., comparable to or lower than those reported in methods previously published.

* Corresponding author.
  E-mail address: massimo.delbubba@unifi.it (M. Del Bubba).
[1] These Authors share the first Authorship.

1. Introduction

On-line solid phase extraction (on-line SPE) coupled with liquid chromatography (LC) is a high analytical throughput technique, suitable to an effective sample preconcentration and matrix removal in the quantification of a wide range of analytes within different contexts, such as environmental and biological fluid analysis, both on untreated and pre-treated samples [1,2]. This technique is commonly applied in combination with triple quadrupole tandem mass spectrometry (QqQ) for quantitative analysis, due to the overall superior performances of this latter detection mode compared to UV or other mass analyzers [3].

The development of an on-line SPE-LC-QqQ method is highly complex due to the need to select sorbent phases suitable for retaining target analytes and to make analytes desorption compatible with subsequent chromatographic separation. When the investigated molecules contained strong hydrophobic moieties, sorbent phases based on silica silanized with alkyl (e.g., C18) [4] or aromatic (e.g., phenyl) [5] groups in combination with reversed phase (RP) chromatography produced satisfactory results, guaranteeing a good analyte retention without effects of breakthrough, nor analyte over-retention. However, if target analytes cover a wide range of physicochemical properties and one or more polar moieties are present in the molecule, the use of sorbents providing multiple interactions (i.e., π-π, dipole-dipole, hydrogen bond, and hydrophobic interactions) is recommended, since they offer a very high retention capacity towards a broad spectrum of target compounds [6]. Due to this, multiple interaction sorbents require in most cases high percentages of organic solvent for their detachment, thus making this desorption strategy poorly compatible with the retention of polar analytes on RP chromatographic stationary phases. On the other hand, the use of eluents suitable for RP-LC results in poor focus of analytes in the chromatographic column and/or presence of carryover effects [7,8].

In order to maintain an RP-LC compatible with the on-line SPE, an alternative desorption strategy relies on the organic desorption followed by post-cartridge dilution (OD-PCD), which consists in the elution of the cartridge with a low volume (tens of μL) of organic solvent and the post-cartridge dilution with an aqueous solvent, as similar as possible to the initial chromatographic conditions [9–11]. The post-cartridge dilution significantly improves the focusing of polar analytes at the top of the column, which otherwise would not be retained or underwent to chromatographic distortions, such as peak broadening and/or splitting.

An alternative strategy for using on-line multiple interaction SPE is its combination with hydrophilic interaction liquid chromatography (HILIC), which is compatible with the use of high percentages of organic solvent at the beginning of the elution gradient [12,13]. However, HILIC is characterized by complex retention mechanisms and a poor reproducibility of retention times with even small variations of mobile phase modifiers (e.g., buffer strength and pH), thus resulting in a kind of "on–off chromatography" for some compounds and unpredictable chromatographic behaviours compared to RP [14].

On the other hand, during the desorption step the elution strength of the aqueous mobile phase can be improved by increasing the temperature of the eluent, which weakens the non-covalent bonds responsible for analyte retention and increases the elution strength of water, due to the lowering of its permittivity [15]. Accordingly, in this study, thermally assisted desorption (TD) was investigated for the first time in on-line SPE-LC-QqQ under RP conditions, using multiple interaction sorbents and water as SPE eluent. This desorption strategy was tested on a model group of structurally heterogeneous analytes comprising 34 human gut nutrimetabolites characterized by (i) a wide range of physicochemical properties, (ii) different physiological conjugations (i.e., glucuronides and sulfonates), and (iii) structural isomerism (i.e., isobars), thus representing a challenging analytical issue. In detail, the chromatographic separation of the model analytes was investigated using different RP stationary phases and eluent combinations and finally optimized focusing on the resolution of critical isobaric groups. Then, TD was studied in comparison with (i) OD-PCD and (ii) conventional

desorption (i.e. using the optimized elution gradient without heating), demonstrating the much better performance of this novel desorption approach in terms of analyte focusing in the analytical column and sensitivity. Finally, the TD method was evaluated for matrix effect and recovery in serum and urine, as relevant applications in nutrimetabolomics [16].

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade water, methanol and acetonitrile were obtained from Carlo Erba (Milan, Italy). Ultrapure water (resistivity >18 MΩ cm) was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Formic acid (HCOOH, MS grade, purity ≥99.0%) and trifluoroacetic acid (CF$_3$COOH, MS grade, purity ≥99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. The chromatographic columns used in this study were the endcapped pellicular Kinetex C18, biphenyl (BP), and phenyl-hexyl (P–H) columns (Phenomenex, Torrance, CA, USA), 100 × 3 mm, particle size 2.6 μm, each. The SPE cartridges Isolute ENV+ and Oasis HLB used in this study were obtained from Biotage (Uppsala, Sweden) and Waters (Milford, MA, USA), respectively. These sorbents were selected based on information regarding their thermal stability (up to 160 °C), given by the manufacturers. Full details of the two cartridges are reported in Table S1 of the Supplementary materials.

2.2. Target analytes and preparation of stock and working solutions

The target compounds of this study (TRC, Ontario, Canada) belonged to the following metabolites categories: hydroxybenzenes, benzoic acids, cinnamic acids, urolithins, stilbenoids, and hippuric acids. Their full name, abbreviation, and physicochemical properties are reported in Table S2 of the Supplementary materials. Luteolin (LUT) and sinapic acid (SIN), used as internal standards, were purchased from Extrasynthese (Genay, France).

Standard stock solutions of individual compounds were prepared by accurate weighing and dissolution in LC-MS grade methanol to give a concentration of 1 g L^{-1}, apart from EA, for which a 0.1 g L^{-1} standard stock solution was prepared due to its lower solubility. A multi-compound standard solution at a concentration of 10 mg L^{-1} of each compound was prepared by proper dilution of the standard stock solutions in LC-MS grade methanol. The latter was further diluted with LC-MS water up to (i) 250 μg L^{-1} for direct injection (DI) analysis, and (ii) 25 μg L^{-1} for the optimization of the on-line SPE method, as operative standard solutions. The standard stock solutions and the 10 mg L^{-1} solution were stored in the dark at −20 °C, whereas the operative solutions were stored in the dark at +4 °C.

2.3. Instrumental configurations tested

Instrumental analysis was performed on a Shimadzu (Kyoto, Japan) chromatographic system coupled with a 5500 QTrap mass spectrometer (Sciex, Framingham, MA, USA), equipped with a Turbo V ESI source (see Section S3 of the Supplementary materials for source dependent parameters). Source and compound dependent parameters were optimized as respectively described in section S3 and Table S3 of the Supplementary materials. QqQ analysis was carried out using multiple reaction monitoring (MRM) in negative ionization mode. The chromatographic analyses were conducted using Analyst (version 1.6.2) and Multiquant (version 3.0.2) softwares (Sciex).

The optimization of the on-line SPE extraction process on multiple interaction sorbents was carried out by investigating three different desorption approaches (see Figs. S1 A–C of the Supplementary materials), i.e. (A) conventional desorption by exposing the cartridge to the optimized chromatographic gradient, without any heating, (B) OD-PCD,

and (C) TD.

All these on-line SPE-LC configurations used a low pressure gradient quaternary pump Nexera X2 LC-30AD (pump 1) and one isocratic pump LC-20AD XR, devoted to the on-line SPE procedure (pump 2). An additional pump (pump 3) is used in the OD-PCD configuration to manage a make-up solvent (i.e., acidified LC-MS grade water), according to the configuration reported in Fig. S1 B. A CTO/20AC thermostatted column compartment equipped with the analytical column, a SIL-30AC auto-injector equipped with a 2-mL sample loop, a DGU-20A 5R degassing unit and a CBM-20A module controller were also used. On-line SPE was performed using a two-position six-port switching valve (model HT, Vici, Schenkon, Switzerland) housing the sorbent cartridge. In the TD configuration (Fig. S1 C), two experimental devices, schematically illustrated in Fig. S2 of the Supplementary materials were tested. TD system 1 (Fig. S2 A) consisted of a stainless-steel block inserted in a Teflon housing and equipped with four holes in which are inserted four 50W/240V cylindrical heaters (RS Components, San Giovanni Milanese, Italy). A further through hole is the housing for the online cartridge. The heaters are connected to a controller model M3 (Ascon Tecnologic S.r.l., Pavia, Italy) that allows for setting and measuring the temperature reached in the housing of the SPE cartridge, thanks to the presence of a thermocouple placed in contact with the stainless-steel block. TD system 2 (Fig. S2 B) consisted of a Shimadzu GC-2010 gas chromatograph, suitably modified to allow the positioning of SPE cartridge and tubing inside the oven.

2.4. Optimization of the chromatographic analysis

Since the model set of target analytes includes several isobaric pairs, their separation was carefully investigated testing different stationary phases. The aforementioned pellicular columns C18, BP, and P–H were selected based on literature data (i.e., C18) and phase-analyte interactions (i.e., steric and π-π). One-variable-at-time chromatographic optimization was performed by investigating eluent composition and flow rates, as described in Table S4 of the Supplementary materials. The chromatographic resolution (see Equation S1 of the Supplementary materials) has been used for evaluating the separation of the critical isobaric pairs.

2.5. Evaluation of the on-line SPE configurations

Preliminarily, to test the applicability of the TD configuration, it was investigated the occurrence of degradation phenomena of target analytes due to their exposure at high temperatures. In particular, these tests were performed in triplicate by placing vials containing the independent aliquots of operative standard solutions (see section 2.2) in a stainless-steel holder and heating at temperatures of 90 and 120 °C, for a total time of 5.5 min (i.e., the same exposure time that analytes underwent during the SPE procedure, as described in section 2.8). Afterwards, the solutions were directly injected and the mean chromatographic area obtained for each analyte (A_T) was compared to that determined by direct injection of the standard solution not exposed to the heating (A), in order to evaluate the extent of thermal degradation as the percentage ratio A_T/A.

The three on-line SPE configurations (Fig. S1) were investigated keeping constant the optimized chromatographic conditions. Desorption temperatures of 60, 90, and 120 °C and methanol volumes of 50 and 100 μL were tested for TD and OD-PCD configurations, respectively.

The performance of the extraction and desorption procedures were evaluated in terms of (i) peak shape and, when possible, (ii) extraction efficiency (EE%, Equation (1)) on all the 34 human gut metabolites, using the aforementioned operative standard solutions at 25 μg L^{-1}.

$$EE\% = \left(\frac{A_{SPE}}{A_{DI}} \bullet 100 \right) \tag{1}$$

A_{SPE} and A_{DI} are the mean chromatographic areas (n = 3) obtained under on-line SPE configuration and DI, respectively. Within day and between day precision associated to EE% was also assessed.

For each human gut metabolite, instrumental detection (iLODs) and quantitation limits (iLOQs) were determined by applying the best performing on-line SPE-TD-LC-MS/MS method (i.e. the TD system 2 set at 120 °C) on operative standard solutions. To this aim, Equation (2) was used, where σ_b is the standard deviation of the procedural blank and S is the slope of the calibration curve in ultrapure water [17]. The instrumental linearity range was also determined, using iLOQ as the lower calibration level.

$$iLOD \ (iLOQ) = \frac{3.3 \ (10) \bullet \sigma_b}{S} \tag{2}$$

2.6. Sample preparation

Serum and urine samples were collected within a randomized, two-arm, acute intervention trial on ten volunteers, who were orally administered a single dose of bilberry or blueberry (i.e., *Vaccinium myrtillus* and *Vaccinium corymbosum*) supplement. Before analysis, serum and urine aliquots were thawed and pre-treated as described elsewhere [18]. For the evaluation of the figure of merits of the analytical method and for the quantitative analysis, pooled samples of serum and urine were prepared separately using the biofluids of all the volunteers, regardless of the treatment administered.

2.7. Figures of merit of the best performing on-line SPE-TD-LC-MS/MS method on biofluids

The performances of the method were evaluated in real samples in terms of percentages of matrix effect (ME%), apparent recovery (AR%), recovery (R%), as well as method detection (MDLs), and quantification limits (MQLs) [17]. These assessments were performed on a pool of on-line SPE eluate fractions from the loading of urine and serum pool aliquots, recovered at the outlet of the analytical column and corresponding to peak-free retention time intervals [7].

ME% was determined using Equation (3):

$$ME\% = \left(\frac{S_{matrix}}{S_{solvent}} \bullet 100 \right) - 100 \tag{3}$$

where S_{matrix} and $S_{solvent}$ are the slopes of calibration lines of each metabolite and internal standards, in matrix and in LC-MS water, respectively.

AR% values of each metabolite and internal standards were evaluated on the aforementioned eluate spiked at two concentration levels (i.e., 25 and 50 μg L^{-1}), analysed as described below (section 2.8), and calculated according to Equation (4), where A_{matrix} is the chromatographic area in spiked eluate pool and $A_{solvent}$ is the chromatographic area in LC-MS water. These samples were analysed from run-to-run over 3 days to estimate a "between day" precision.

$$AR\% = \left(\frac{A_{matrix}}{A_{solvent}} \bullet 100 \right) \tag{4}$$

R % was defined using Equation (5):

$$R\% = AR \ \% - ME \ \% \tag{5}$$

MDLs and MQLs were assessed according to Equation (6):

$$MDL \ (MQL) = \frac{iLOD \ (iLOQ)}{AR\%} \tag{6}$$

2.8. Operative on-line SPE-LC-MS/MS conditions

In the operative conditions, the TD system 2 was adopted, performing the sample analysis as follows. The 2-mL loop was filled with

500 μL standard solution/sample at 10 μL s^{-1} (filling time of the loop equal to 50 s). Meanwhile, the six-port switching valve was set on the loading position, allowing the analytical column to be conditioned by pump 1 with acidified water (0.1% HCOOH) and 2% of acetonitrile, at a flow rate of 0.7 mL min^{-1}. When the loop was filled, the elution program of pump 1 started. Simultaneously, pump 2 delivered a solution of ultrapure water acidified at pH = 2 with HCl at 1.0 mL m^{-1} in for 2.8 min, thus loading the 500 μL of the standard solution/sample onto the Isolute ENV + cartridge. During the loading phase, the cartridge was kept at 25 °C. Subsequently, the temperature of the oven that houses the cartridge was brought to 120 °C in about 20 s and maintained for 5.5 min before switching the valve to the injection position, and back-flushing the SPE cartridge by pump 1 for the entire gradient program, thus allowing the transfer of target analytes to the analytical column. The chromatographic conditions are summarized hereafter. Chromatographic column: Kinetex P–H; eluent "A": acidic LC-MS grade water (0.1% HCOOH); eluent "B": acidic methanol (0.1% HCOOH); eluent "C": acidic acetonitrile (0.1% HCOOH); flow rate: 0.70 mL min^{-1}; temperature: 40 °C; elution gradient: eluent "C" 2.0% for the entire duration of the analysis; eluent "B" percentage 2.0% for 1 min followed by its increase at 2% min^{-1} for 13 min, 5% min^{-1} for 7.5 min, and final 100% of "B" in 3 min. The system is then brought to the initial conditions in 0.5 min for re-equilibrating the analytical column.

3. Results and discussion

3.1. Chromatographic optimization

Three superficially porous stationary phases (i.e., Kinetex C18, BP, and P–H), characterized by the same geometry, were tested (section 2.1). Chromatographic optimization was performed by evaluating the setups reported in Table S4, using the 250 μg L^{-1} operative solution and an injection volume equal to 50 μL (i.e., injecting 12.5 ng of each analyte). The acidity of mobile phases was kept constant at pH = 2.7 ± 0.1 with 0.1% formic acid, to promote the neutral speciation of the target analytes (see Table S2), without affecting their electrospray ionization. In this latter regard, when stronger acidity was used (e.g., 1% formic acid or 0.1% trifluoroacetic acid), a relevant drop of sensitivity (data not shown) was observed as elsewhere reported in literature [19].

The target set of analytes included five isobaric groups: 2,3-DBA/2,5-DBA, TRES/UR-A, iFA-SULF/FA-SULF, QUE-3-GLU/QUE-7-GLU, and UR-A-GLU/iUR-A-GLU/TRES-GLU. Since the first three groups showed chromatographic resolutions (R$_{Chrom}$, Equation S1) > 1.5 in all the investigated setups, the discussion of the optimization procedure focused only on UR-A-GLU/iUR-A-GLU/TRES-GLU and QUE-3-GLU/QUE-7-GLU groups. Table S4 also reports the R$_{Chrom}$ values of the critical isobaric groups for each investigated experimental condition. Setups 1–2 (i.e., C18 combined with methanol as organic eluent) were not able to resolve any of the isobaric groups, while the replacement of C18 with BP increased the retention of all isobaric groups and particularly of TRES-GLU, thus providing its resolution from the urolithin derivatives (R$_{Chrom}$ = 2.4). The use of P–H gave rise to results very similar to those achieved with C18, i.e. without any baseline separation of the isobaric groups (data not shown).

The organic strength of mobile phase was increased using acetonitrile instead of methanol, evaluating also different flow rates (from 0.4 to 0.6 mL min^{-1}), thus achieving lower analyte retention and a general improvement of R$_{Chrom}$. These setups (i.e., 4-to-6 in Table S4) effectively allowed for obtaining baseline separations of the two urolithins and between iUR-A-GLU and TRES-GLU. In addition, an increase in the separation of the quercetin derivatives was observed, being the best results obtained at 0.5 mL min^{-1} (setup 5, R$_{chrom}$ = 0.6). These findings could be addressed to the multiple selectivity mechanisms exerted by the BP stationary phase, such as shape, polarizability, and polarity of the target analytes [20].

In an attempt to improve the separation of pairs only partially

resolved in setup 5, the P–H was also tested. In fact, based on literature [21], this stationary phase should have a greater capacity to adopt different spatial orientations of the aromatic ring, thus potentially giving rise to a wider interaction capacity of the stationary phase with the target analytes, compared to BP column. The results of configurations 7 and 8 clearly showed the complete loss of resolution of urolithin derivatives, which were however well separated from resveratrol, while quercetin derivatives remained only partially resolved. Further tests on the P–H column focused on the use of methanol and methanol/acetonitrile eluents. As illustrated in Table S4, the use of a constant 2% of acetonitrile within a gradient water/methanol as described in Section 2.5, provided a good compromise of the R$_{Chrom}$ values, leading to a satisfying resolution for all critical isobaric groups, when the flow rate of 0.7 mL min^{-1} was adopted (see also Fig. S3 of the Supplementary materials). This finding is justified by the combination of the organic strength of methanol towards analytes and the π-π disrupting properties of acetonitrile towards P–H, the latter increasing the partition rate to the mobile phase [22]. Further increments of flow rate, probably helpful to improve critical resolutions, were not allowed due to an incomplete spray formation in the ion source. Thus, setup 11 was confirmed as the operative chromatographic method to be coupled with on-line SPE.

3.2. Development of the on-line SPE-LC-MS/MS method

Once the optimal chromatographic conditions were identified and the system configured for on-line SPE, the three different desorption strategies described in Fig. S1 were investigated to maximize the EE% of target metabolites. In detail, on-line SPE was studied performing (i) the conventional desorption, i.e. using the optimized LC elution gradient keeping the sorbent at ambient temperature (Fig. S1 A), (ii) the OD-PCD (Fig. S1 B), and (iii) the TD using the same optimized gradient elution adopted for the conventional desorption (Fig. S1 C), investigating three temperature levels (i.e., 60, 90, and 120 °C). Furthermore, the two commercially available multiple interaction cartridges Isolute ENV+ and Oasis HLB, fully described in Table S1, were evaluated comparatively.

Under the experimental conditions adopted for loading the sample onto the on-line cartridge (i.e., ultrapure water acidified at pH = 2 with HCl at flow rate of 1.0 mL min^{-1} for 2.8 min), breakthrough phenomena were never highlighted in both sorbents, thus evidencing the strong retention capacity of the multiple interaction phases tested here against the target analytes.

3.2.1. Conventional desorption

The development of the on-line SPE method was initially investigated by adopting the configuration reported in Fig. S1 A, i.e., exposing the SPE cartridge to the flow of the mobile phase from pump 1, for the entire chromatographic run. Desorption performance of both cartridges were studied in these experimental conditions for all target analytes, highlighting very similar behaviours of the two stationary phases. In detail, the increase in the organic strength along the elution gradient was not enough rapid to promote the focusing of target analytes, thus generating broad and unresolved elution profiles, particularly for more polar compounds. As an example, Fig. S4 of the Supplementary materials illustrates the desorption from the Isolute ENV + cartridge of four representative analytes (i.e., CA-GLU, 5-FQA, 2,3-DBA, and UR-A). These compounds were chosen to cover almost the entire chromatographic window and a wide range of values of the logarithm of octanol-water partition constants (Log D) at the loading and desorption pH values (pH = 2–3, see Table S2 of the Supplementary materials). These findings are in agreement with results elsewhere reported for the desorption from multiple interaction sorbents of gut metabolites similar to the ones here investigated [23,24]. In fact, in these papers, the analytes detachment required the use of 100% organic solvents, while the chromatographic gradient adopted here reached 100% organic in about 26 min, thus determining the aforementioned distortion of the

chromatographic profile and making unfeasible any evaluation of the analytes EE% using these on-line SPE conditions.

3.2.2. Organic desorption with post-cartridge dilution

Based on literature information [9,10], to solve the issue of over-retention of analytes on on-line SPE sorbents, the OD-PCD configuration illustrated in Fig. S1 B was also tested on both Isolute ENV+ and Oasis HLB cartridges. Using this configuration, it is possible to promote partition equilibria of the analytes more in favour of the eluent by introducing a small volume of organic solvent into the cartridge. Then, the analytes can be focused at the top of the analytical column by diluting the cartridge eluate with an aqueous solution. Despite the apparent simplicity of this approach, a high complexity is experienced, since it is essential to optimize the volumes of both desorption organic solvent and focusing aqueous solution, as well as the speed and timing with which they are dispensed. Tests were conducted for this purpose, trying to identify the best compromise among an efficient desorption of the analytes, their focusing and the sensitivity of the on-line SPE method, which is of course affected by the post-cartridge dilution. As a general consideration, better recoveries were obtained using the Isolute ENV + cartridge. The best results were obtained by configuring pump 1 to deliver 100 μL (i.e., approximately the void volume of the cartridge) of methanol acidified with 0.1% formic acid (i.e., 0.14 min at 0.7 mL min^{-1}) in the cartridge, immediately after the loading step. At the end of this dosage, the cartridge was exposed for 1 min to the starting chromatographic conditions (i.e., 2% acetonitrile, 2% methanol, and 96% water) while the post-cartridge dilution was performed by dosing 100 μL of 0.1% aqueous formic acid (make-up solvent) with pump 3 (i.e., 1 min at 0.1 mL min^{-1}). Afterwards, the valve was rotated to the loading position (Fig. S1 B, left side) and the elution gradient started as described in section 2.8.

Compared to the previous configuration, the analytes desorption and focusing was significantly improved, even though the peak shapes were still broad in comparison with DI (see Fig. S5 of the Supplementary materials). Moreover, the chromatographic resolution of UR-A-GLU/iUR-A-GLU was drastically worsened and QUE-3-GLU/QUE-7-GLU co-eluted completely (see Fig. S6 A-B of the Supplementary materials).

In spite of these drawbacks, the optimized OD-PCD approach allowed for calculating the recovery of all target metabolites for both cartridges. Fig. 1 illustrates the individual recoveries obtained for the four representative metabolites previously selected (Fig. 1 A) and the mean recovery of all target analytes (Fig. 1 B), highlighting the better performances of the Isolute ENV + cartridge. The lower recovery obtained with the Oasis HLB sorbent should be attributed to an insufficient desorption capacity of the eluent used, rather than a lower retention capacity of the sorbent during the loading phase, as no breakthrough was observed, as mentioned above. A lower recovery of Oasis HLB compared to Isolute ENV+ was elsewhere reported for the off-line SPE of various phenolic compounds in aqueous matrices, although no clear interpretation of this result was given [25].

In detail, EE% values achieved with the Oasis HLB and the Isolute ENV+ was 26 ± 15 (range: 2–56) and 40 ± 23 (range: 10–106), respectively. Therefore, even under the best OD-PCD conditions, i.e. with the ENV + cartridge, the EE% was unsatisfactory, with three analytes (i.e., VA, TRES, and TRES-SULF) showing values < 20% and those related to further eleven compounds included in the range 20–30%.

3.2.3. Thermally assisted desorption

Due to the problems encountered with the OD-PCD strategy, it was decided to adopt an innovative SPE approach compared to what has been reported in the literature so far, investigating the effect of temperature on the partition between the sorbent phase and the eluent, during the desorption step from the cartridge. Using a desorption strategy based on the increase in the cartridge temperature, the detachment of analytes from the sorbent should be facilitated due to the (i) weakening of the non-covalent bonds between the analyte and the sorbent phase and (ii) decrease in the permittivity of water, which brings its eluent strength closer to that of an alcohol at room temperature [26, 27].

The thermal degradability tests carried out at 90 and 120 °C on the 34 target metabolites highlighted in all cases a good stability (see Fig. S7 of the Supplementary materials), thus allowing to test the TD strategy for the whole model group of analytes.

The TD system 1 (Fig. S2 A) was the first to be tested showing better desorption with the ENV + than the Oasis HLB, similarly to what was observed with the OD-PCD approach. Fig. 2 shows the results obtained for the ENV + cartridge with the TD system 1 set at the temperatures of 60 °C and 90 °C. These results refer to the recovery of the aforementioned four representative gut metabolites (Fig. 2 A) and to the mean recovery of the whole group of target analytes (Fig. 2 B). Moreover, Fig. S8 A-D of the Supplementary materials illustrates for the same four individual analytes the peak shape obtained under the best experimental conditions, i.e. 90 °C. The heating of the cartridge improved the detachment of the analytes from the cartridge, whose peak shape appears much more homogeneous and narrower, compared to the profile observed for the OD-PCD technique, even though the resolutions of the critical isobaric pairs UR-A-GLU/iUR-A-GLU and QUE-3-GLU/QUE-7-GLU were partially or totally lost (see Fig. S6 C-D). The recovery was also improved, with a clear effect of the temperature increase from 60 °C to 90 °C, the latter corresponding to a mean recovery value for all analytes of about 50%. No further significant increase in the desorption temperature (i.e., up to 120–130 °C) was unfortunately possible with the TD system 1, because the target temperature was reached too slowly to allow a prompt detachment of the analytes, probably due to the characteristics of the cylindrical heaters used for the implementation of the device.

To overcome this drawback the TD system 2 (Fig. S2 B) was tested. Based on the results achieved with the TD system 1, the temperatures of
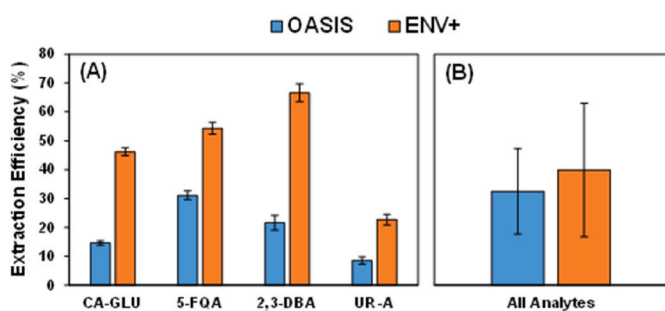

Fig. 1. Extraction efficiency of selected analytes (box A) and all analytes (mean value, box B) from the OASIS HLB and Isolute ENV + sorbents, using the organic desorption with post-cartridge dilution. Error bars represent standard deviation.
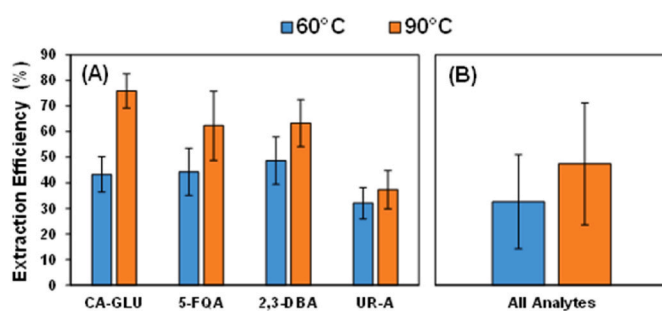

Fig. 2. Extraction efficiency of selected analytes (box A) and all analytes (mean value, box B) from the Isolute ENV + sorbent, using the thermally assisted desorption system 1 set at 60 and 90 °C. Error bars represent standard deviation.

90 °C and 120 °C were investigated on both sorbents also in the TD system 2. A prompt detachment of analytes and narrow desorption peaks were obtained also with this desorption system (Fig. S9 of the Supplementary materials). Furthermore, similarly to findings obtained with OD-PCD and TD system 1, higher recoveries were achieved with the ENV + than the OASIS HLB (Fig. 3). The results obtained as a function of the temperature applied to the cartridge confirmed the trend previously observed on the TD system 1. In detail, at the desorption temperature of 120 °C, the mean extraction efficiency was 95 ± 25, with only two analytes characterized by EE% values less than 60%, i.e. QUE-3-SULF and FA-SULF, both exhibiting values of about 30% (see Table S5 of the Supplementary materials).

Also with this desorption strategy the critical resolutions of the isobaric pairs UR-A-GLU/iUR-A-GLU and QUE-3-GLU/QUE-7-GLU were not maintained (see Fig. S6 E-F), thus highlighting this behaviour as a general drawback of the translation of the DI method into the online SPE one. The TD on-line SPE-LC-MS/MS chromatogram of the operative standard solution under the selected experimental conditions is reported in Fig. S10 of the Supplementary materials, while Fig. 4 shows the comparison between the MRM chromatograms obtained by TD on-line SPE (Fig. 4 A-F) and DI (Fig. 4 G-L) for the four aforementioned representative analytes (i.e., CA-GLU, 2,3-DBA, 5-FQA, and UR-A), and the critical isobaric resolutions UR-A-GLU/iUR-A-GLU/TRES-GLU and QUE-7-GLU/QUE-3-GLU.

The experimental conditions based on the use of the ENV + cartridge and the TD system 2 heated at 120 °C were considered as optimal and therefore tested for the evaluation of the instrumental performances. The vast majority of metabolites showed iLODs and iLOQs of tens-hundreds of ng/L, with the sole exceptions of VA, EA, and 2,3-HYBA, which exhibited sensitivities of few µg L^{-1} (Table S5). The linearity range was investigated from the iLOQ values up to 500 µg L^{-1}, showing good R^2 values (0.9962–0.9998) for the entire set of metabolites (Table S5).

3.3. Method validation in urine and serum samples

The on-line SPE method using the TD strategy 2 on the ENV + cartridge was validated for the analysis of target gut metabolites and internal standards in serum and urine samples from an intervention study on bilberry and blueberry intake [18]. The validation involved the
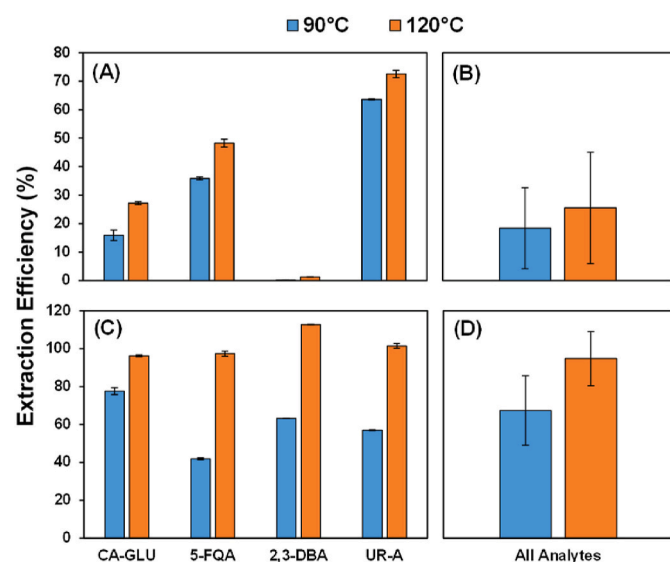

Fig. 3. Extraction efficiency of selected analytes (boxes A and C) and all analytes (mean value, boxes B and D) from OASIS HLB (boxes A and B) and Isolute ENV+ (boxes C and D) sorbents, using the thermally assisted desorption system 2 set at 90 and 120 °C. Error bars represent standard deviation.

parameters mentioned in section 2.7, i.e., ME%, MDLs, MQLs, AR%, and R% (see Table S6 of the Supplementary materials). Fig. S11 of the Supplementary materials illustrates the chromatographic profile of some analytes, obtained under the proposed experimental conditions in spiked ultrapure water (Fig. S11 A-C), urine (Fig. S11 D-F), and serum (Fig. S11 G-I).

3.3.1. Urine samples

Urine samples showed signal suppression rather than an increase in most cases. However, the effects due to the alteration of ionization efficiency in the ESI source was mostly negligible, being |ME| ≤ 20% in almost all cases. The only significant exceptions were TRES and TRES-SULF with a suppressive effect of 26%, and above all SAL, which exhibited a signal suppression of 38%.

AR% values, evaluated on target metabolites and internal standards at 25 µg L^{-1} and 50 µg L^{-1} (levels I and II in Table S6, respectively), were characterized by a high precision, being the relative standard deviation (RSD) associated with independent replicates (n = 3) in all cases ≤8%. Furthermore, the values of AR% determined for each compound at the two spiking levels were very similar and therefore a mean AR% was calculated and used to assess the values of R%. Overall, R% values ranged from 31% to 118%. In more detail, for 26 out of 35 analytes (note that for co-eluting QUE-3-GLU and QUE-7-GLU, a cumulative recovery was calculated) R% was included in the pseudo-quantitative range of 80–120%, while 7 compounds were recovered between 60% and 79%, and the remaining 2 in the range 30–59%.

The method sensitivity was found similar to the instrumental one, due to the limited effect of the matrix on both on-line SPE and ionization efficiency in the ESI source. Accordingly, MDLs and MQLs were mostly included between tens and hundreds of ng L^{-1}, with the very few above-mentioned exceptions of VA, EA, and 2,3-DBA (MQLs of 2.0, 7.2, and 4.9 µg L^{-1}, respectively, see Table S6), to which QUE-3-SULF is added (MQL = 1.3 µg L^{-1}), due to its low value of AR%. Hence, method sensitivity here achieved is optimal for the quantification of target analytes in biological fluids, where their concentrations commonly range from nM to µM levels [28].

It is interesting to compare the performances of the on-line SPE method here optimized with SPE protocols elsewhere reported that use mixed mode sorbents (i.e., hydroxylated styrene–divinylbenzene copolymer and styrene–divinylbenzene copolymer functionalized with N-vinylpyrrolidone). One paper focusing on off-line SPE shares with our study FA, VA, HVA, CA, 3-HPAA, UR-A, UR-B, and SIN, for which only R % values were however reported [23].

The comparison highlighted better performances of our method, with the only exception of HVA. Furthermore, the gain in analysis time obtained with the on-line method was significant, since the analysis of one sample took less than 30 min against about 3 h of the off-line one.

3.3.2. Serum samples

Unlike urine, serum samples were mostly characterized by a signal enhancement. However, the effect of the matrix on compound ionization was in almost all cases negligible (i.e., |ME| ≤ 20%), being α-HA and CA-GLU the only exceptions, with values of matrix effect of about 28% and 33%, respectively (Table S6). This finding is particularly interesting in light of the high complexity of the serum matrix and the relative simplicity of the proposed on-line protocol.

Similar to the results shown in urine, the precision associated with the three independent replicates of the AR% assessment was also very high in serum, the RSD% values being ≤6% (Table S6).

Overall, values of R% ranged from 13% to 113%. In more detail, only 10 out of 35 analytes showed R% ≥ 70%, whereas 11 analytes were recovered between 40% and 69% and the remaining 15 below 40%. These findings affected also the overall method sensitivity for serum analysis, evidencing in some cases MQLs in the 1–2 µg L^{-1} range (i.e., 3-MC, 2,5-DBA, 3-HPAA, and QUE-3-SULF) and even higher, such as VA (4.7 µg L^{-1}), EA (23.1 µg L^{-1}), and 2,3-DBA (20.4 µg L^{-1}). However, the
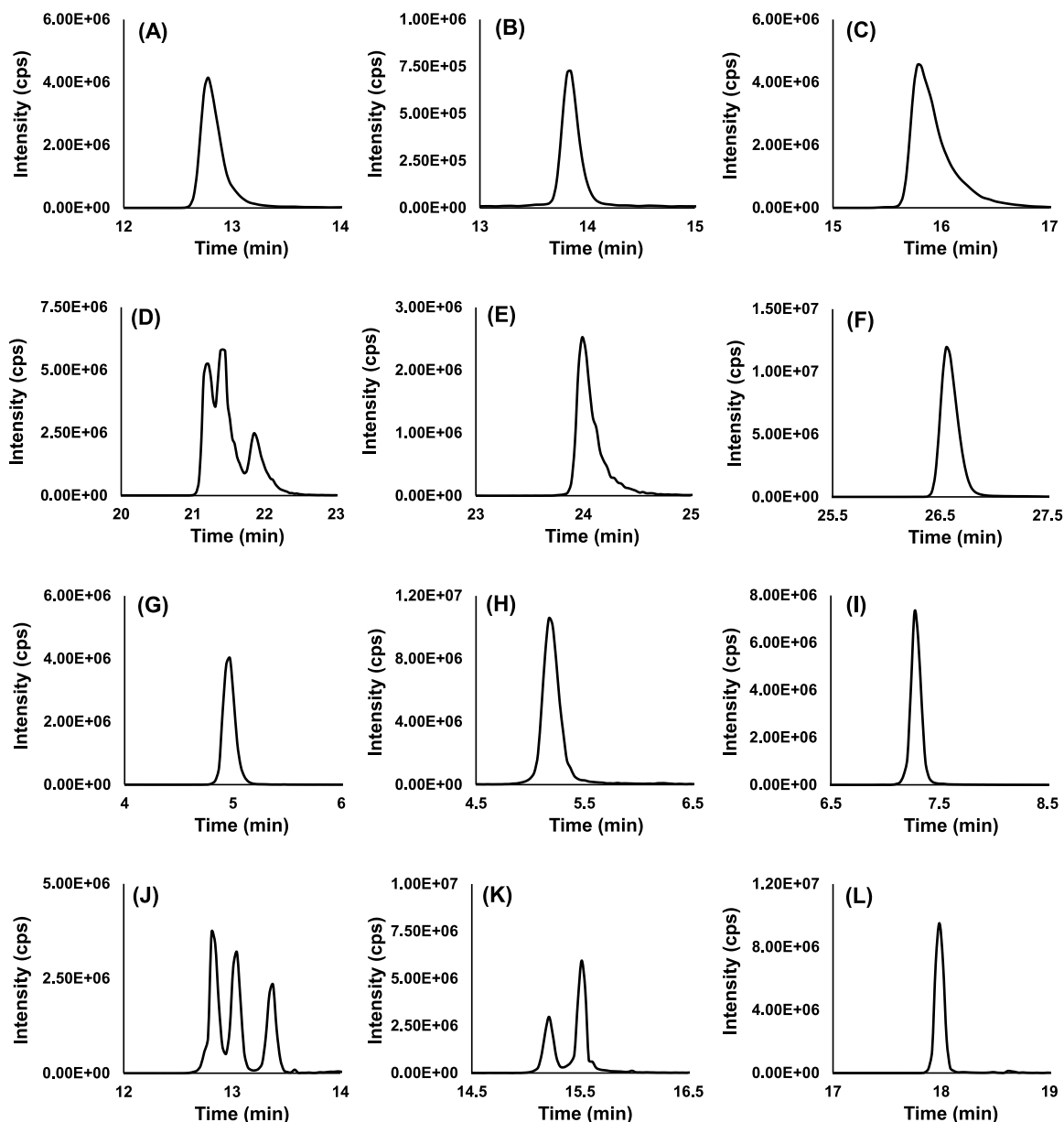
Fig. 4. MRM chromatograms obtained by the thermally assisted desorption (system 2 set at 120 °C) on-line SPE-LC (A–F) in comparison with the direct injection (G–L) for the four representative analytes CA-GLU (A–G), 2,3-DBA (B–H), 5-FQA (C–I), and UR-A (F–L), and the critical isobaric resolutions UR-A-GLU/iUR-A-GLU/TRES-GLU (D–J) and QUE-7-GLU/QUE-3-GLU (E–K).

other analytes exhibited comparable or slightly higher MQLs compared to urine, while remaining in the tens to hundreds ng L^{-1} range.

Two papers have been previously published with a focus on the analysis of nutrimetabolites in serum using off-line SPE protocols based on mixed mode copolymer sorbents and therefore comparable with our study [23,29]. Fig. 5 illustrates by mean of boxplots the comparison of the values of ME%, R%, and MQL for the 8 (Ref. 22) and 23 (Ref. 28) compounds that are in common with this study. The proposed method generally involved a signal enhancement instead of the suppressive trend highlighted elsewhere (Fig. 5 A). However, the matrix effect values were mostly within the range ±20%, which is commonly considered negligible [17], thus evidencing the effectiveness of this kind of sorbents in the removal of the matrix constituents. The trend of R% (Fig. 5 B) found in this study was characterized by a greater dispersion and a lower overall average, especially in comparison with the method proposed by González-Domínguez and co-workers [29], which is more robust from this point of view. On the other hand, despite the higher

recoveries reported elsewhere, MQLs found in our study (Fig. 5C) were much less dispersed and far lower than those achieved by the off-line SPE approaches under consideration. This result should be attributed to the straightforward procedure here proposed by using on-line SPE on 500 μL of sample aliquots, rather than to higher instrumental performance, as the sensitivity of the mass spectrometer used in this study is comparable to that of the instruments used in the other researches.

4. Conclusions

For the first time, temperature-assisted desorption was investigated for the detachment of analytes strongly retained on mixed mode polymer sorbents, demonstrating the feasibility of this analytical strategy in the on-line SPE approach and on highly complex biological matrices such as urine and serum. In this regard, it should be noted that after method optimization the ENV + cartridge was used for about 150 injections showing the same chromatographic profile and extraction
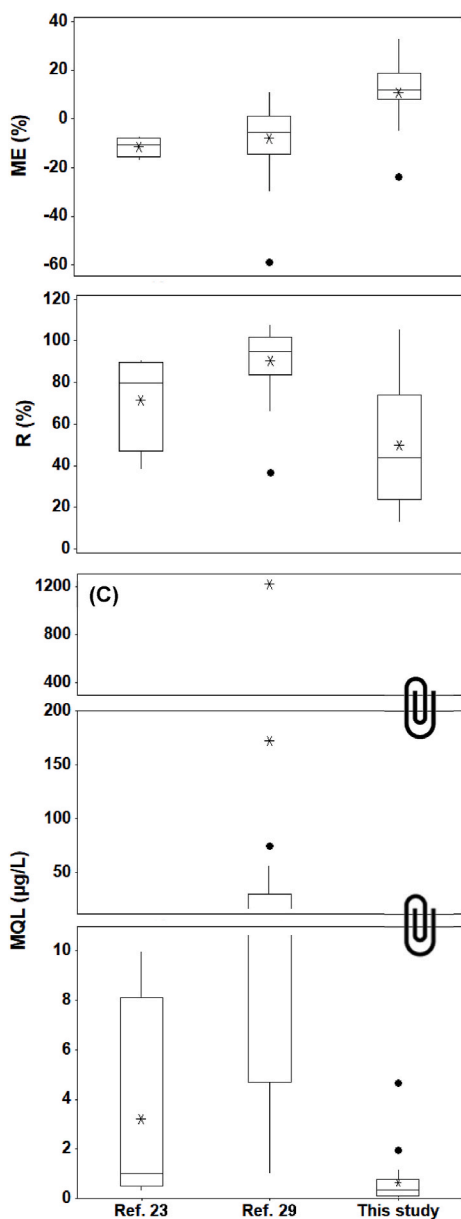
Fig. 5. Boxplot of the values of ME%, R% and MQL determined in Ref. 23 and 29, in comparison to this study. The boxplots refer respectively to 8 (Ref. 22) and 23 (Ref. 28) compounds in common with this study. The boxes represent the interquartile range, while the line is the median; lower and upper whiskers extend to the maximum or minimum data point within 1.5 box height from the top or bottom of the box; asterisk is the mean; black round symbols are the outliers.

performance.

This research is also the first on-line SPE study on a large group of nutrimetabolites with very different physicochemical properties. The optimized method is suitable for the RP-LC analysis of gut metabolites in targeted metabolomics platforms. However, when the chromatography optimized for DI analysis was translated in on-line SPE (using either OD-PCD or TD approaches) the critical resolutions UR-A-GLU/iUR-A-GLU and QUE-3-GLU/QUE-7-GLU were partially or totally lost. Thus, to increase the discrimination of these isobaric pairs the chromatography should be re-optimized.

CRediT authorship contribution statement

Lapo Renai: Methodology, Validation, Investigation, Writing –

original draft. **Luca Marzullo:** Formal analysis, Investigation, Resources. **Giulia Bonaccorso:** Investigation. **Serena Orlandini:** Visualization. **Fulvio Mattivi:** Writing – review & editing. **Maria Concetta Bruzzoniti:** Writing – review & editing. **Massimo Del Bubba:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341429.

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