New and Vintage Solutions To Enhance the Plasma Metabolome Coverage by LC-ESI-MS Untargeted Metabolomics: The Not-So- Simple Process of Method Performance Evaluation

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17 **ABSTRACT**

18 Although LC-MS untargeted metabolomics continues to expand into exiting research domains,
19 methodological issues have not been solved yet by the definition of unbiased, standardized and
20 globally accepted analytical protocols. In the present study, the response of the plasma metabolome
21 coverage to specific methodological choices of the sample preparation (two SPE technologies, three
22 sample-tosolvent dilution ratios) and the LC-ESI-MS data acquisition steps of the metabolomics
23 workflow (four RP columns, four elution solvent combinations, two solvent quality grades,
24 postcolumn modification of the mobile phase) was investigated in a pragmatic and decision tree-like
25 performance evaluation strategy. Quality control samples, reference plasma and human plasma from
26 a real nutrimetabolomic study were used for intermethod comparisons. Uni- and multivariate data
27 analysis approaches were independently applied. The highest method performance was obtained by
28 combining the plasma hybrid extraction with the highest solvent proportion during sample
29 preparation, the use of a RP column compatible with 100% aqueous polar phase (Atlantis T3), and
30 the ESI enhancement by using UHPLC-MS purity grade methanol as both organic phase and
31 postcolumn modifier. Results led to the following considerations: submit plasma samples to hybrid
32 extraction for removal of interfering components to minimize the major sample-dependent matrix
33 effects; avoid solvent evaporation following sample extraction if loss in detection and peak shape
34 distortion of early eluting metabolites are not noticed; opt for a RP column for superior retention of
35 highly polar species when analysis fractionation is not feasible; use ultrahigh quality grade solvents
36 and “vintage” analytical tricks such as postcolumn organic enrichment of the mobile phase to enhance
37 ESI efficiency. The final proposed protocol offers an example of how novel and old-fashioned
38 analytical solutions may fruitfully cohabit in untargeted metabolomics protocols.

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Untargeted metabolomics continues to expand to exciting life science application domains1−3 fuelled by progress in high-resolution liquid chromatography mass spectrometry (LCMS), bioinformatics tools for data processing, and by recent huge investments (http://commonfund.nih.gov/Metabolomics/ fundedresearch). However, methodological maturity has not yet been reached, thus hindering field's progress and application to epidemiology.4 The growing number of metabolomic studies and initiatives aimed to solve methodological concerns (Figure S-1, Supporting Information)5−13 and set objective criteria for method optimization14,15 have not yet converged into the definition of unbiased, standardized and globally accepted analytical protocols. Certainly, the ambitious challenge of a comprehensive read-out of the metabolome complicates method performance evaluation, since the clear-cut criteria established for the validation of targeted LC-MS/MS protocols16 are hardly applicable to this different approach (nonquantitative, and nonspecific for a selected cluster of metabolites). Like any targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) bioanalysis, untargeted liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) metabolomics suffers from matrix effects responsible for ion suppression phenomena, which in turn hamper metabolome coverage, mass accuracy and analytical reproducibility. 17,18 Particularly, in the case of complex biomatrices (i.e., blood fluids), inadequate sample preparation and LC-MS data acquisition procedures are known major sources of ion suppression,8,10−12 but discussion on how to overcome specific methodological hindrances related to these two steps of the workflow still appears fairly limbic. Regarding sample preparation, for instance, the vast majority of the attempts to optimize the sample extraction procedures still focus on a partial removal of blood-interfering components (namely proteins),19,20 instead of searching for the most satisfactory compromise between the exhaustive removal of all the species responsible for matrix effects and nonselective metabolite extraction (metabolome coverage). 19−21 In addition, an ideal sample preparation method should be as simple as possible, with the minimal number of steps required. In contrast, laborious and potentially "risky" steps such as sample evaporation and

65 reconstitution prior to LC-MS analysis are often incorporated into the procedure, but evidence of the
66 real benefits obtained has not yet been established in untargeted studies.22 Regarding the LC-MS
67 data acquisition phase, the main challenge lies in the detection of thousands of known and unknown
68 components in a wide range of chemistries, molecular masses, dynamic concentration range and MS
69 responses, possibly in a single analysis. Reverse phase (RP)-LC is considered the most suitable
70 analytical tool for ESI-MS highthroughput analysis of heterogeneous samples,23 but the nonretention
71 of highly polar metabolites generally poses a chromatographic challenge (coelution of many chemical
72 species at the solvent front, competition in the use of the energy available for ionization at the source
73 of the MS, thus mutual ion suppression phenomena). The choice to resort to dual analysis (i.e.,
74 RP/HILIC) has been much widespread so far,11 with no apparent consideration of its environmental
75 impact (generation of hazardous chemical waste) and reduced applicability to largescale studies
76 (sample, time, labor and solvent consumption). Nevertheless, new RP column formats designed to
77 promote superior retention of highly polar ionic species are currently available, and may lead to a
78 satisfactory separation of a wide metabolite chemical diversity without the need for ion pairing agents
79 or analysis fractionation. Furthermore, the combination of new chromatographic solutions with the
80 careful selection of mobile phases and the use of "vintage" analytical tricks traditionally used to
81 enhance ESI efficiency in RP-LC (i.e., the postcolumn organic modification of mobile phases) has
82 not been explored so far in untargeted metabolomics.8,17,24−27 In the present study, the response of
83 the plasma metabolome coverage to specific methodological choices of the sample preparation (two
84 SPE technologies, three sample-to-solvent dilution ratios) and the LC-ESI-MS data adquisition steps
85 of the metabolomics workflow (four RP columns, four elution solvent combinations, two solvent
86 quality grades, postcolumn modification of the mobile phase) was investigated in a pragmatic and
87 decision tree-like performance evaluation strategy. Quality control samples, reference plasma and
88 human plasma from a real nutrimetabolomic study were used for intermethod comparisons, injected
89 in a batch-designed randomized sequence order. To overcome the not-so-simple process of method

90 performance evaluation in untargeted metabolomics, uni- and multivariate data analysis approaches
91 were independently applied.

92 **EXPERIMENTAL SECTION**

93 Figure 1 shows the different methodological scenarios tested in a simplified decision tree-like
94 flowchart. The main criteria and tools used for method performance evaluation are listed in Table 1.

95 Samples. Three sample types were used for comparison among the different methodological set-ups:
96 aqueous standard metabolite mixes, reference plasma, and individual human plasma samples
97 collected during a dietary intervention study.

98 An aqueous standard mix (QC2) composed by metabolites representative of the plasma metabolome
99 chemical variety was prepared, including six amino acids, two carnitines, three organic acids, two
100 acyl glycine conjugates, an ester of acetic acid and choline, two fatty acids and two flavonoid
101 compounds (details in Table S-1, Supporting Information). A second standard mix of twenty-one
102 highly polar metabolites (LogP $\ll$ 0, POL mix) was also prepared for comparison between RP and
103 HILIC chromatographic performance (details in Table S-1, Supporting Information). Aqueous
104 solutions of isotopically labeled compounds were also prepared for use as internal (IS) and external
105 standard (ES) mix during sample extraction (details in the Supporting Information).

106 Commercial reference plasma (Sigma-Aldrich, St Louis, MO) was used in a first-pass method
107 performance evaluation, to avoid biological variability among compared samples. Plasma was
108 alternatively spiked with milli-Q water (unspiked) and with the QC2 mix (1 and 5 µg/mL final
109 standard concentrations), to evaluate the capacity of the tested methodological set-ups to detect
110 known expected quantitative differences among spiked and unspiked reference samples. Human
111 plasma samples from a previously described nutritional intervention study12,28 were finally used to
112 assess the influence of specific methodological choices in a real metabolomic study (plasma collected
113 before and 2-h after an acute intake of a cocoa-based drink). The choice of the case study was not

114 casual, but pushed by criteria of logic functionality, to easily orient the evaluation of the different
115 methodological options according to the detection of expected biomarkers of cocoa intake. Sample
116 Extraction Performance Evaluation. 96-well Plate SPE Technologies. Plasma (50 μL) was subjected
117 to an inplate hybrid extraction method consisting of deproteinization by acidic solvent precipitation
118 (ACN 1% FA) followed by phospholipid SPE-mediated removal.12 Two 96-well plates were
119 independently used for sample SPE (plate 1: Ostro, Waters; plate 2: Phree, Phenomenex) according
120 to the respective manufacturer's suggestions. Sample cleanup from matrix effects was first evaluated
121 by comparing the residual total phospholipid profile of plasma samples extracted through the two 96-
122 well plates, compared to samples subjected to traditional deproteinization techniques alone (organic
123 extraction with ACN, MeOH, MeOH:EtOH 1:1 v/v).12 This was carried out by monitoring the XIC
124 at m/z 184.070−184.075 (ESI+ mode), corresponding to the yield of trimethylammonium-ethyl
125 phosphate cations released from the residual (lyso)phospholipids still present in the extracts, when
126 applying high-energy declustering potential for in-source fragmentation (+90 V). Experiments of
127 permanent postcolumn infusion of QC2 standard metabolites were then used. Compared to more
128 conventional approaches (i.e., individual standards addition), the postcolumn infusion technique is in
129 fact a more suitable tool for the evaluation of matrix effects in untargeted LC-MS protocols, because
130 it gives information on signal suppression/ enhancement phenomena occurring throughout the whole
131 chromatogram, independently of a specific retention time29,30 (details in the Supporting
132 Information). The recovery of QC2 standard metabolites spiked in plasma, the capacity to detect
133 subtle quantitative differences in reference plasma (raw versus spiked) and metabolomic changes in
134 human plasma following an acute cocoa intake were also used to compare the sample extraction
135 performance of the two plates. To check for extraction reproducibility, repeated independent
136 extractions (≥3 technical replicates) of each biological sample were carried out. In all cases, aqueous
137 isotopically labeled standard mix were added to the sample matrices before (IS) and after (ES) the
138 extraction (10 μg/mL final standard concentration), to check for extraction reproducibility and

139 analytical stability during LC-MS data adquisition. The successful removal of phospholipids from
140 the samples was also confirmed by including a lysophosphatidylcholine molecule (1-O-stearoyl-
141 snglycero- 3-phosphocholine) in the IS mix (negative control). Sample-to-Solvent Dilution Ratio
142 (v/v). Three sample-tosolvent dilution ratios (1:6, 1:9 and 1:12 v/v) were then evaluated during the
143 extraction process, according to the plate manufacturer suggestions. For this experiment, reference
144 plasma samples (raw, spiked) were used, and the three dilution ratios were evaluated in terms of the
145 extraction efficiency of the standard metabolites spiked in plasma (peak detection, peak intensities,
146 peak intensity changes among sample classes, $p < 0.05$), the extraction reproducibility (CV of peak
147 intensities among technical replicates) and the shape of the early eluting peaks according to the
148 organic percentage of the extracts. Chromatographic and Electrospray Ionization Performance
149 Evaluation. LC Columns, Elution Solvents and Postcolumn Organic Modification. In line with the
150 choice of maintaining a single-step analysis, four RP column formats with different silica chemistries
151 were first compared (details in "Columns", Supporting Information), ranging from a traditional
152 HPLC C18-based stationary phase to three (U)HPLC columns designed for superior retention of
153 highly polar ionic species. Column performance was evaluated upon a no. ≥450 randomized sample
154 injections, by comparing the retention capacity (mean RT and k factor of QC2 standard metabolites
155 showing a wide range of partition coefficients, log P −3.19 − +8.23) and the technical reproducibility
156 of the analysis (column back pressure, across-run pressure stability, RT reproducibility by retention
157 time CV).

158 For ESI enhancement purposes, four mobile phase combinations were then tested (ESI+ and ESI−),
159 differing in the nature of the aqueous [A] and organic [B] phases and in the solvent quality (LC-MS
160 versus ultrahigh performance (UHP)LC-MS purity grade) (details in "Mobile Phases", Supporting
161 Information). Second, the impact of a postcolumn organic enrichment of the mobile phase was also
162 tested by adding a 100 μL/min MeOH flow at the LC-MS interface, so as to reduce the aqueous
163 proportion reaching the MS detector and enhance ESI efficiency (15% increase of organic final

164 concentration). Metabolite detection (peak signal intensities), peak shape, width and symmetry, and
165 the extent of real-life plasma metabolome changes detected in the metabolomics case study were used
166 for intermethod comparison of ESI performance. The performance of the most successful RP-LC-
167 MS setup was finally compared to the use of a HILIC column (XBridge BEH Amide column 100 ×
168 2.1 mm, 4 μm, Waters) in terms of retention capacity and reproducibility, and MS detection sensitivity
169 (POL mix). The not-mentioned operating conditions for LC-ESI-qToFMS data acquisition were set
170 as previously described12 (Supporting Information). To avoid possible bias in intermethod
171 comparison, all extracts resulting from different sample preparation were analyzed in a unique batch-
172 designed and randomized run sequence order, except when not feasible (i.e., comparison among
173 different RP columns, analysis with or without the postcolumn organic modifier). Samples were
174 subdivided into homogeneous sub-batches (∼10 injections each) separated by the regular analysis of
175 QC samples (∼30% of the total runs). For system suitability check, the following quality control (QC)
176 samples were analyzed throughout the data acquisition: QC1, Milli-Q water samples; QC2 (5 μg/mL
177 final standard concentration); QC3, randomly selected biosamples repeatedly injected along the
178 sequence of analysis. Blank extractions (solvent only) were also injected at the beginning of the
179 sequence, to verify any eventual solvent-dependent mass features not to be considered during
180 comparative analysis. Prior to analysis, a minimum of two QC2 and ten biological samples were
181 injected, respectively to check for system suitability and for system conditioning with the sample
182 matrix. Analyst QS 2.0 software was used for data acquisition and system control (Applied
183 Biosystems, Foster City, CA, USA). Chemometric Data Analysis. Before statistical analysis, data
184 preprocessing was carried out using MarkerView 1.2.1. software (AB Sciex, Toronto, Ontario,
185 Canada). Data were log10 transformed and Pareto-scaled to approach a normal distribution, and data
186 quality assured as previously described.12 Data reproducibility was a common requirement for all
187 the conditions tested.31 The analytical variability across the runs was then evaluated by monitoring
188 the standard metabolite components of QC2 samples injected over time. Since several of the tested

189 parameters may modify LC and MS response of the QC2 metabolites (i.e., RT shifts and variation in
190 mass signal intensities depending on the different columns, mobile phases and organic modifier), the
191 entire analysis sequence was divided into smaller unmatchable sequences of experiments, and data
192 quality assurance was carried out in intraexperiment separate evaluations (details in "System
193 suitability check", Supporting Information). Subsequently, UVA (t test for pairwise sample
194 comparisons, $p < 0.05$) and MVA (PCA, PCA-DA, OPLS-DA) were applied to the different data sets
195 for comparative analysis, as previously described,12,32 by using both commercial (MarkerView TM
196 1.2.1., AB Sciex, Toronto, Ontario, Canada; SIMCA P+ v13, Umetrics, Umea˚, Sweden) and online
197 tools (XCMS).33 PCA score plots, S- and SUS-plots, box plots and relational diagrams were helpful
198 for data comparison and visualization.34 Finally, the in-house R-based MAIT package35 was used
199 for the computationally assisted identification of significant metabolites up- or down-regulated in the
200 nutrimetabolomic case study.

201 **RESULTS AND DISCUSSION**

202 Table 1 shows an overview ranking of the methodological options tested, according to the proposed
203 evaluation criteria. Sample Extraction Performance Evaluation. 96-well Plate SPE Technologies. The
204 first three criteria used to compare the performance of the two SPE plates were not able to show a
205 clear-cut difference between the sample extraction technologies. In fact, a similar sample cleanup
206 was observed, with negligible levels of residual phospholipids compared to extracts obtained by
207 sample protein precipitation alone (Figure S-2, Supporting Information), resulting in low signal
208 suppression phenomena at the phospholipid elution zone (5.8−8.5 min of the chromatographic run).
209 Similar infusion profiles were also observed, with minimal matrix effects (Figure S-3, Supporting
210 Information). Two suppression zones in the chromatogram (i.e., negative fluctuations in the matrix
211 profiles at ∼0−0.5 and 6−7 min) were more accentuated in extracts obtained by the Ostro plate,
212 possibly due to less efficient removal of salts (front solvent) and late-eluting components from the

213 matrix, respectively. However, none of the mass features that were differentiating the extracts in those
214 specific areas corresponded to metabolites of the infused QC2 mix, and were considered as artifactual
215 features (signals from chemical impurities, background noise). Considering the standards recovery
216 (QC2 metabolites spiked in reference plasma), the use of the Phree plate appeared to be associated
217 with an overall enhanced extraction efficiency (percentage recovery in Table S-2, Supporting
218 Information). However, recoveries varied more depending on the analyte monitored than on the SPE
219 technology used, so not to lead to conclusive results (Figure S-4, Supporting Information). Univariate
220 analysis was then used to compare the two extraction technologies upon the capacity to detect subtle
221 quantitative variation in the plasma metabolome (≤5 μg/mL-scale changes). In this case, the use of
222 the Ostro plate revealed an increased capacity to detect statistically significant differences between
223 raw versus spiked reference plasma (t test results in Table S-2, Supporting Information), suggesting
224 that an apparent reduced metabolite recovery may not necessarily be associated with a loss of
225 biologically relevant information. Multivariate analysis of the data confirmed the presence of relevant
226 differences among sample extracts, according to the extraction procedure. Figure 2 shows a PCA
227 scores plot of raw versus spiked reference plasma, extracted by the SPE plate 1 versus 2, and analyzed
228 in presence versus absence of the postcolumn mobile phase modification (ESI+ mode). Because the
229 first two PCs of the PCA scores space give the direction of the maximum spread of the data, the
230 exploratory analysis clearly showed how the extraction plate and the postcolumn organic
231 modification of the mobile phase were much stronger determinants of sample variation than the
232 differences among sample classes (similar results in ESI− mode, Figure S-5, Supporting Information).
233 To obtain a definitive comparison, the nutritional case study was used to assess the extent of
234 metabolomic changes detected in real plasma, according to the sample extraction technology used
235 during sample preparation (supervised multivariate analysis). No difference was observed in the
236 number of significant mass features of cocoa intake between the two extraction plates, with a high
237 extent of data overlapping (Figure S-6, Supporting Information). The most common metabolites

238 expected to be up- and down-regulated following the acute intake of cocoa (i.e., theobromine,
239 caffeine, decanoylcarnitine) were detected in both types of plasma extracts, and confirmed our
240 previous observations.12,27 However, more robust (O)PLS-DA models were obtained by submitting
241 samples to extraction with the Ostro plate (p-value, R and Q intercepts in Table S-3, Supporting
242 Information). For these reasons, and because the final goal of the application of any metabolomic
243 method is to highlight even subtle metabolite up- and down-regulation in comparative analysis, the
244 Ostro plate finally gave the greatest extraction performance (Table 1).

245 Sample-to-Solvent Dilution Ratio (v/v). Table S-4 (Supporting Information) shows the comparative
246 analysis between the different sample-to-solvent dilution ratios tested during extraction. The three
247 ratios were first compared according to the capacity to extract known expected mass features from
248 spiked plasma samples (peak detection, peak intensities and CV depending on the organic proportion
249 during extraction) and the capacity to highlight subtle differences between raw and spiked samples (t
250 test pairwise comparisons, p-value). The use of the highest organic proportion during sample
251 preparation (1:12 sample-to-solvent ratio, v/v) was not associated with a significant loss of metabolite
252 detection, possibly expected due to the greater final sample dilution. The highest solvent proportion
253 was associated with a higher extractability of almost all the monitored compounds (ratio of peak
254 intensities, Table S-4, Supporting Information), and appeared to slightly improve extraction
255 reproducibility across samples (lower peak intensity CV, Table S-4, Supporting Information). Finally,
256 no peak shape distortion of the early eluting metabolites was noticed to hamper mass feature detection
257 (Figure S-7, Supporting Information), although no solvent replacement was carried out by
258 evaporation prior to LC-MS analysis.

259 For all these reasons, the use of the highest sample dilution factor was considered the best
260 compromise between extractive capacity, sample dilution and final organic percentage in the extracts
261 (Table 1). Chromatographic and Electrospray Ionization Performance Evaluation. RP Columns. The
262 use of the Atlantis T3 column provided the best chromatographic performance in terms of superior

263 retention of polar metabolites (2-fold higher k factors than by using the traditional C18-based column,
264 for compounds with log P < 0) with negligible effects on the elution of the most nonpolar species
265 monitored and so on the global analysis speed. The column also showed good retention time
266 reproducibility (RT variation < 5 s) (Table S-5, Supporting Information), lower column back pressure
267 and higher across-run pressure stability (pressure variation <5% across up to 800 injections) (Table
268 S-6, Supporting Information). In comparison to the use of a hydrophilic interaction chromatography
269 (HILIC) column (BEH Amide, Waters), the retention capacity and resolution power of polar
270 compounds with structural isomerism (i.e., glucose and fructose-6-phosphate) was low (k < 1),
271 consistent with the RP nature of the stationary phase. However, peak detection enhancement obtained
272 by HILIC chromatography was neither directly related to the increased retention capacity nor
273 common to all the polar species monitored (i.e., higher sensitivity for D-fructose 1,6-bisphosphate,
274 citric, L-lactic, oxalic, maleic, pyruvic and propionic acids by using RP separation, as shown in Table
275 S-5, Supporting Information). Consequently, these findings did not support a clear-cut improvement
276 by the use of fractionation analysis (RP/HILIC), and confirmed that many factors other than column
277 chemistry can be modulated in order to enhance sensitivity in MS. Elution Solvents. Although the
278 effect on signal response was found to be compound dependent, the use of nonacidified MeOH as
279 organic phase [B] gave the strongest molecular ion signal intensities, in both ionization modes (Figure
280 S-8, Supporting Information). Particularly, in ESI− mode, the use of MeOH increased peak intensities
281 up to 25-fold compared to the reference organic phase used in previously validated protocols (ACN
282 0.1% FA).32 Moreover, peak broadening effects putatively expected with the use of MeOH36 were
283 negligible in respect of the gain in peak intensity enhancement observed (Figure S-8, Supporting
284 Information), and were neither shared by all the monitored metabolites nor detrimental for peak
285 detection during data preprocessing. Finally, the use of UHPLC-MS quality grade MeOH gave
286 between a 2- and 37-fold increase in peak intensities, compared to the ACN-based organic phase
287 (Figure S-8, Supporting Information, ESI− mode). Postcolumn Mobile Phase Modification. The

288 organic enrichment of the mobile phase prior to the MS detection was a strong determinant of sample
289 variation (Figure 2). In fact, it consistently associated with a significant enhancement of mass signal
290 intensities for all the monitored metabolites eluting up to two-thirds of the chromatogram (Figure 3),
291 leading to an increased capacity to detect up to 1 μg/mL-scale changes between raw and spiked
292 reference plasma samples. A decrease in mass signal intensities was not common among the late-
293 eluting metabolites, and loss of peak detection did not occur at the concentration range monitored (1
294 −5 μg/mL), suggesting that the diluting effect of the extra volume of mobile phase infused did not
295 dramatically affect metabolite detection (dilution effect). Furthermore, peak broadening (putatively
296 expected due to dead volume introduction) was again negligible and not associated with the loss of
297 peak detection. Finally, the postcolumn organic modification of the mobile phase also enhanced the
298 detection of significant biological changes occurring in the plasma metabolome after an acute dietary
299 intervention. To verify the impact of using this analytical solution in the enhancement of ESI
300 efficiency and the detection of subtle metabolomic differences among real sample classes, the number
301 of biomarkers detected in plasma following an acute intake of cocoa, in the absence or presence of
302 the postcolumn modification of the mobile phase, was compared. Figure 4 shows a SUS-plot
303 comparing the significant mass features detected by the two models (model 1, with mobile phase
304 modification; model 2, without mobile phase modification). As shown in the figure, although the
305 strongest biomarkers of cocoa intake were detected independently on the use of postcolumn addition
306 (mass features found on the plot diagonal represent shared biomarkers, i.e., theobromine and
307 caffeine), other previously described metabolites were uniquely detected through the organic
308 modification of the mobile phase (mass features far from the diagonal, i.e. upregulation of
309 glycochenodeoxycholic acid, down-regulation of L- (iso)leucine).12,37 Nevertheless, the majority of
310 the cocoaassociated discriminant mass features observed following postcolumn organic modification
311 kept unidentified, hampering to fully evaluate the extent of method performance enhancement.

312 **CONCLUSION**

313 Although being excellent samples for assessing pathophysiological deviations in both endogenous
314 and exogenous metabolism, blood fluids are as informative as challenging samples.38 Inadequate
315 preparation and LC-MS analysis of these complex biomatrices are major sources of ion suppression
316 phenomena, which in turn adversely affect the most crucial prerequisite for untargeted metabolomics,
317 such as metabolome coverage, mass accuracy, and data reproducibility. However, there is still no
318 consensus on how to overcome specific analytical hindrances and make large-scale untargeted studies
319 feasible. Even the method performance evaluation process is not fully standardized, due to the
320 difficulty in defining validation criteria to cover the specific problems associated with untargeted
321 analysis of (mostly unknown) compounds. Although the concept of method optimization is not viable
322 in untargeted metabolomics, the joint finishing of sample extraction procedure, chromatographic
323 separation and electrospray ionization still forms the necessary basis to develop a successful and
324 robust LC-ESI-qToF-MS (qToF, quadrupole time-of-flight) methodology, and reach a balanced
325 compromise between metabolome coverage and feasibility. In the present work, we investigated the
326 response of the plasma metabolome coverage to specific methodological choices related to sample
327 preparation, chromatographic separation and ESI process enhancement procedures in RP-LC-MS
328 data adquisition. The best sample extraction performance was obtained when combining sample
329 hybrid extraction with high organic proportion. During sample preparation, both a partial removal of
330 undesired interference or a too drastic nonselective cleanup of the samples would lead to poor
331 metabolome coverage and compromise the detection of subtle metabolite variation in comparative
332 analysis.8,19−21 In a previous work, we observed that the selective removal of phospholipid-based
333 matrix effects is a more successful alternative for plasma sample preparation than deproteinization
334 alone12 (the "less is more" concept). In the present work, two in-plate hybrid extraction technologies
335 were compared, and conventional criteria for extraction efficiency evaluation were not definitive in
336 making lean toward one or the other plasma preparation procedure. Nevertheless, the observed
337 findings suggested that an apparent reduced recovery of specific standard metabolites is not

338 necessarily associated with a loss of biologically relevant information, confirming that established
339 criteria for the optimization and validation of targeted LC-MS/ MS bioanalysis should be integrated
340 with untargeted approaches for method performance evaluation, in order to get closer to the real
341 objectives of the analytical protocols. Although no solvent removal/replacement was carried out after
342 sample extraction and prior to LC-MS analysis, neither significant loss of metabolite detection nor
343 early eluting peak shape distortion were observed in our study, suggesting that extract evaporation
344 and reconstitution steps may not be truly necessary following the proposed sample preparation
345 protocol. Extract evaporation followed by reconstitution is commonly incorporated in sample
346 preparation procedures, generally justified by solvent removal/replacement and low-sensitivity issues
347 (concentration purposes). However, concerns about introducing these potentially "risky" steps are
348 widely shared (risk of incomplete solubilization of the dry residues and losses of hydrophobic and
349 volatile species, increased ion suppression due to preconcentration of interferents, reduced sample
350 throughput due to considerable time consumption), and no evidence has been produced so far of the
351 actual benefits in terms of metabolome coverage.22 Furthermore, the sample-to-solvent ratio giving
352 the highest final plasma dilution and greatest organic proportion was associated with a higher
353 extractability of the monitored metabolites, without compromising peak detection and shape. The
354 tested ratios were chosen upon the plate manufacturer suggestions and consistently with the challenge
355 of minimizing ion suppression phenomena while bypassing subsequent sample concentration.
356 Although the best dilution ratio is not an absolute parameter to extrapolate for application to different
357 extraction procedures, our findings suggested that sample dilution may be a valuable choice to reduce
358 matrix effects and enhance metabolome coverage. The use of Atlantis T3 RP column compatible with
359 100% initial aqueous phase was then found the best option to maximize retention of polar metabolites.
360 The replacement of acidified ACN with nonacidified MeOH as organic phase [B] gave the strongest
361 molecular ion signal intensities, in both ionization modes. Although acidification of both mobile
362 phases of the elution gradient is a common technique in LC-UV analysis, in order to avoid major

363 baseline disruptions when working at lowabsorbance wavelengths (i.e., 210 nm), this rule does not
364 apply in LC-MS data acquisition, allowing the best binary systems to be chosen in view of the
365 ionization enhancement. Besides showing the best spray solvent characteristics in terms of volatility
366 and surface tension (low dielectric constant and viscosity), MeOH is also known to facilitate lipid
367 elution and avoid accumulation in the column (i.e., as acetone), which may further contribute to the
368 minimization of matrix effects.27 Furthermore, compared to harmful solvents such as ACN, MeOH
369 is a more “environmentally friendly” alternative in terms of workers, processes and environment
370 safety (i.e., ecotoxicity),39 and its replacement in LC applications should be encouraged as a
371 “greener” option.40,41 The purity grade of the solvents used in mobile phase preparation revealed to
372 be an even more crucial factor in mass signal detection, although at least LC-MS purity grade solvents
373 were used. These findings underlined the importance of checking the levels of contaminants in the
374 solvent, such as plasticizers and surfactants, which are very often readily ionizable and can compete
375 for charge with the metabolites.42 Finally, the postcolumn modification of the mobile phase by
376 organic enrichment confirmed to be a pragmatic solution to increase the ionization efficiency without
377 influencing the chromatographic separation, although no applications in RPLC- ESI-MS untargeted
378 metabolomics have been described until now. Other postcolumn solvents apart from methanol were
379 not considered in our flowchart, for several reasons. The use of the same solvent for both mobile
380 phase and postcolumn enrichment of the phase has been suggested as preferable, and the use of
381 nonacidified MeOH as organic phase [B] gave the strongest molecular ion signal intensities in both
382 ESI modes. Both MeOH and ACN are considered as adequate as postchromatography organic
383 modifiers, but they do not differ strongly in decreasing the droplet surface tension in the ESI source,
384 and so in helping ionic evaporation. However, MeOH shows some peculiar advantages in respect to
385 acetonitrile, such as a higher vapor pressure and a lower surface tension, which facilitate even more
386 solvent evaporation. Summarizing, the results of the present work led to the following considerations:
387 plasma samples should be submitted to hybrid extraction for removal of interfering components, to

minimize all the major sample-dependent matrix effects; solvent evaporation following sample extraction may be avoided if no peak shape distortion of early eluting metabolites is noticed; a RP column for superior retention of highly polar species should be chosen when analysis fractionation is not feasible, ESI efficiency may be enhanced by using UHPLC-MS quality grade solvents and "vintage" analytical tricks, such as postcolumn organic enrichment of the mobile phase. The final proposed protocol offers an example of how novel and old-fashioned analytical solutions should fruitfully cohabit in untargeted metabolomics protocols, and deserves consideration for the rapid and simple LC-ESI-MS untargeted fingerprinting of large-scale complex biomatrices. We are aware of the internal limitations of the work. To simplify the process of method performance evaluation, only those factors expected to mostly impact the metabolome coverage (SPE technology, postcolumn organic modification of the mobile phase) where finally tested upon real samples, to compare the capacity to detect known biomarkers of cocoa intake. Thus, several specific comparative analysis were carried out on reference plasma samples only (raw, spiked). Although the standard metabolites monitored were representative of a wide range of chemistries, molecular masses, dynamic concentration range and MS responses (39 metabolites in the QC2/ POL mix, $70 < m/z < 800$, $-9 < \log P < 4$), there is no guarantee that the behavior of the thousands of variables constituting the whole metabolome can be extrapolated from a smaller number of compounds. Second, data on the direct comparison between sample dilution versus evaporation/reconstitution are not presented in this work, so further analysis will be required to get a definitive evaluation.

ASSOCIATED CONTENT

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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416 **Notes**

417 The authors declare no competing financial interest.

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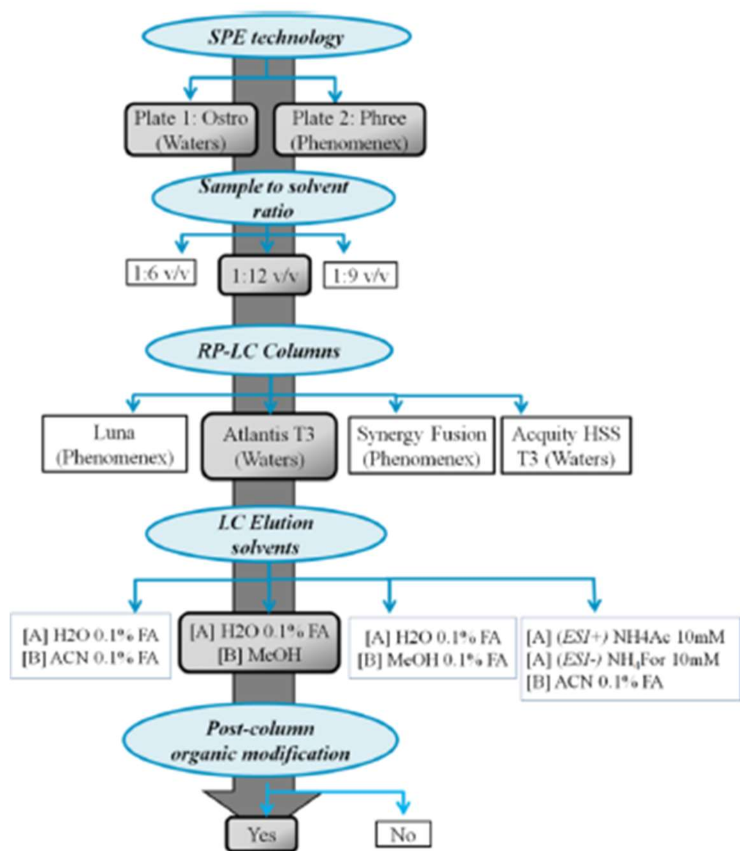
498 **FIGURES**



499 **Figure 1.** Decision tree-like flowchart showing the methodological scenarios tested in the study.

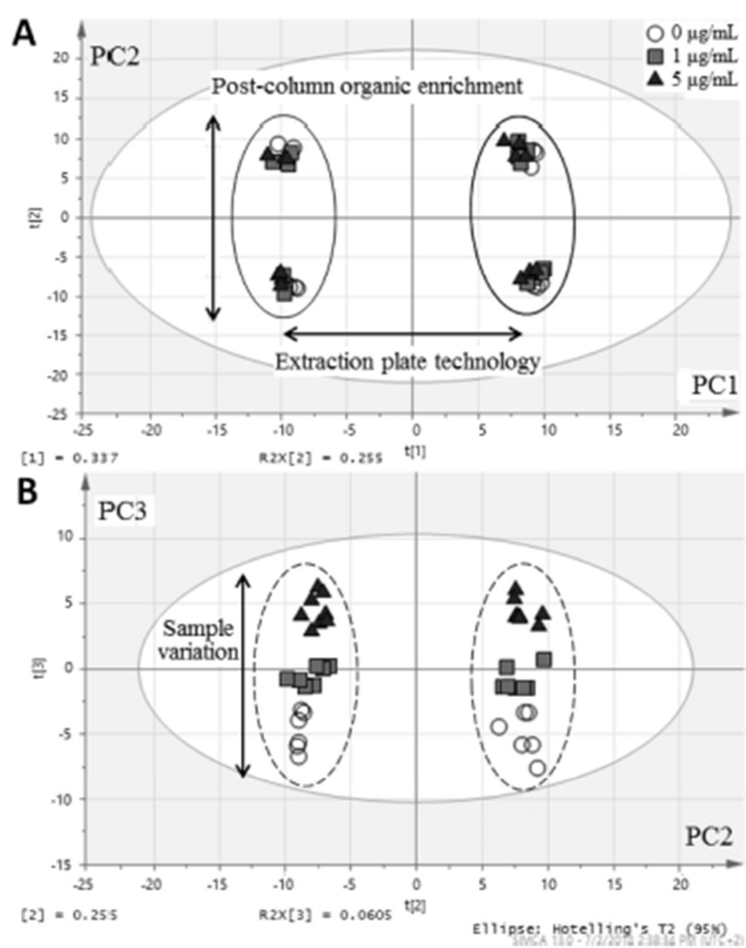


Figure 2. PCA scores plots of plasma samples subjected to extraction by plate 1 versus 2 (discriminated by PC1, solid line circles) and analyzed with or without postcolumn organic modification (discriminated by PC2, dashed line circles); ESI+ mode. Sample classes (plasma unspiked (0) and spiked with QC2 mix at 1 and 5 μg/mL final concentration) were only discriminated by PC3.

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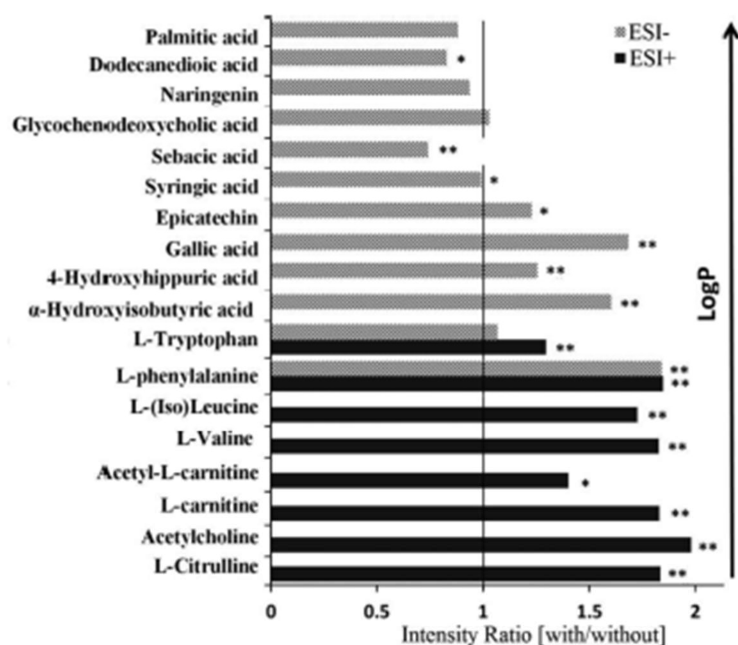

Figure 3. Ratios of mass signal intensities obtained with vs without the use of postcolumn modification of the mobile phase (*, significant differences, $p < 0.05$; **, highly significant differences, $p < 0.01$). Intensities were expressed as mean of five technical replicates. CV ranged from 1 to 21%.

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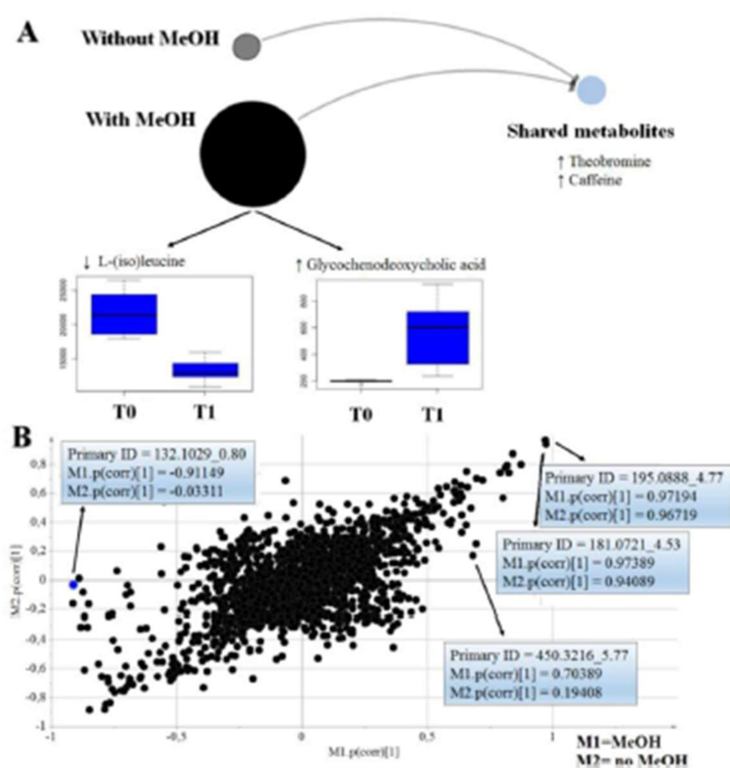

Figure 4. (A) Relational diagram matching the extent of metabolomic changes detected in plasma after an acute dietary intervention by using or not the postcolumn organic modification of the mobile phase (metaXCMS). Uniquely identified metabolites significantly down-(left) and up-regulated (right) following the intervention are shown (boxplots, XCMS). (B) SUS-plot comparing the two models (SIMCA). Mass features far from the diagonal were uniquely detected by the mobile phase modification (model 1).

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TABLES

Table 1. Ranking of the Analytical Options According to the Proposed Evaluation Criteria

	criteria for method comparison	sample type, tools	ranking			
sample extraction performance evaluation	SPE technology		Ostro plate		Phree plate	
	residual total phospholipid profile (XIC at m/z 184.07, ESI+)	reference plasma (raw)	+++		+++	
	residual ion suppression effects (postcolumn infusion experiments[b])	QC2 mix, reference plasma	++		+++	
	standard metabolite recovery (peak intensiy ratios, %[b])	reference plasma (raw, spiked)	++		+++	
	detection of ≤5 μg/mL-scale changes[b,c,d]	reference plasma (raw, spiked)	+++		++	
	detection of real-life metabolomic changes[c,e,f]	plasma from human intervention study	+++		++	
	sample-to-solvent dilution ratio			1:6, v/v	1:9, v/v	1:12, v/v
	metabolite extractability[b,c,d]	reference plasma (raw, spiked)		+	+	++
	early eluting peak shape[b]	reference plasma (raw, spiked)		++	++	++
	technical reproducibility (peak signal intensity CV)[c]	reference plasma (raw, spiked)		++	++	+++
LC and ESI performance evaluation	RP-LC columns		Luna C18	HSS T3	Synergy	Atlantis T3
	retention capacity of highly polar metabolites (k factor[b])	QC2/POL mix, reference plasma (unspiked)	+	++	++	+++
	technical reproducibility (pressure stability, RT reproducibility[b])	QC2 mix, reference plasma (unspiked)	+++	+	+++	+++
	LC elution solvents[g]		System 1	System 2	System 3	System 4
	metabolite detection[c]	QC2 mix, reference plasma (raw, spiked)	++	+++	++++	+
	peak shape, width and symmetry[b]	QC2 mix, reference plasma (raw, spiked)	+++	+++	+++	++
	postcolumn organic modification		yes		no	
	metabolite detection[c]	QC2 mix, reference plasma (raw, spiked)	+++		++	
	detection of real-life metabolomic changes[c,e,f]	plasma from human intervention study	+++		++	

[a]Scores are in the range of + to ++++ against the evaluation criteria. [b]Peak signal intensities, Analyst 2.0 software, Applied Biosystems, Foster City, CA, USA. [c]UVA: t test pairwise comparisons between sample classes (MarkerView TM 1.2.1. software). [d]MVA: PCA, PCA-DA (SIMCA-P+ 13.0 software, Umetrics). [e]MVA: PCA, PCA-DA, (O)PLS-DA (SIMCA-P+ 13.0 software, Umetrics). [f]XCMS online. [g]Mobile phase combination 1 (ESI ±): [A] H_2O 0.1% FA, [B] ACN 0.1% FA. Mobile phase combination 2 (ESI ±): [A] H_2O 0.1% FA, [B] MeOH 0.1% FA. Mobile phase combination 3 (ESI ±): [A] H_2O 0.1% FA, [B] MeOH. Mobile phase combination 4 (ESI+): [A] 10 mM NH4Ac, [B] ACN 0.1% FA. (ESI−): [A] 10 mM NH_4, [B] ACN 0.1% FA.

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