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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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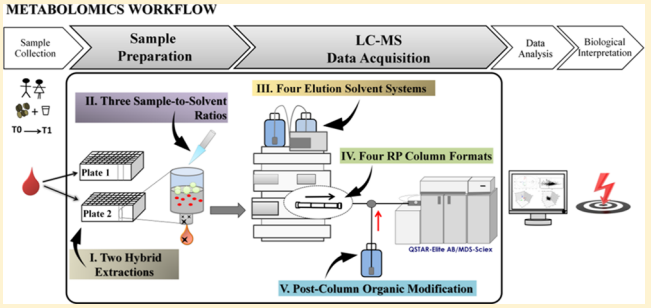
Supporting Information

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



Untargeted metabolomics continues to expand to exciting life science application domains[1−3] fuelled by progress in high-resolution liquid chromatography mass spectrometry (LC-MS), 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 optimization[14,15] 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 protocols[16] are hardly applicable to this different approach (nonquantitative, and nonspecific for a selected cluster of metabolites).

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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 reconstitution prior to LC-MS analysis are often incorporated into the procedure, but evidence of the real benefits obtained has not yet been established in untargeted studies.[22]

Regarding the LC-MS data acquisition phase, the main challenge lies in the detection of thousands of known and unknown components in a wide range of chemistries, molecular masses, dynamic concentration range and MS responses, possibly in a single analysis. Reverse phase (RP)-LC is considered the most suitable analytical tool for ESI-MS high-throughput analysis of heterogeneous samples,[23] but the nonretention of highly polar metabolites generally poses a chromatographic challenge (coelution of many chemical species at the solvent front, competition in the use of the energy available for ionization at the source of the MS, thus mutual ion suppression phenomena). The choice to resort to dual analysis (i.e., RP/HILIC) has been much widespread so far,[11] with no apparent consideration of its environmental impact (generation of hazardous chemical waste) and reduced applicability to large-scale studies (sample, time, labor and solvent consumption). Nevertheless, new RP column formats designed to promote superior retention of highly polar ionic species are currently available, and may lead to a satisfactory separation of a wide metabolite chemical diversity without the need for ion pairing agents or analysis fractionation. Furthermore, the combination of new chromatographic solutions with the careful selection of mobile phases and the use of "vintage" analytical tricks traditionally used to enhance ESI efficiency in RP-LC (i.e., the postcolumn organic modification of mobile phases) has not been explored so far in untargeted metabolomics.[8,17,24−27]

In the present study, the response of the plasma metabolome coverage to specific methodological choices of the sample preparation (two SPE technologies, three sample-to-solvent dilution ratios) and the LC-ESI-MS data adquisition steps of the metabolomics workflow (four RP columns, four elution solvent combinations, two solvent quality grades, postcolumn modification of the mobile phase) was investigated in a pragmatic and decision tree-like performance evaluation strategy. Quality control samples, reference plasma and human plasma from a real nutrimetabolomic study were used for intermethod comparisons, injected in a batch-designed randomized sequence order. To overcome the not-so-simple process of method performance evaluation in untargeted metabolomics, uni- and multivariate data analysis approaches were independently applied.

EXPERIMENTAL SECTION

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

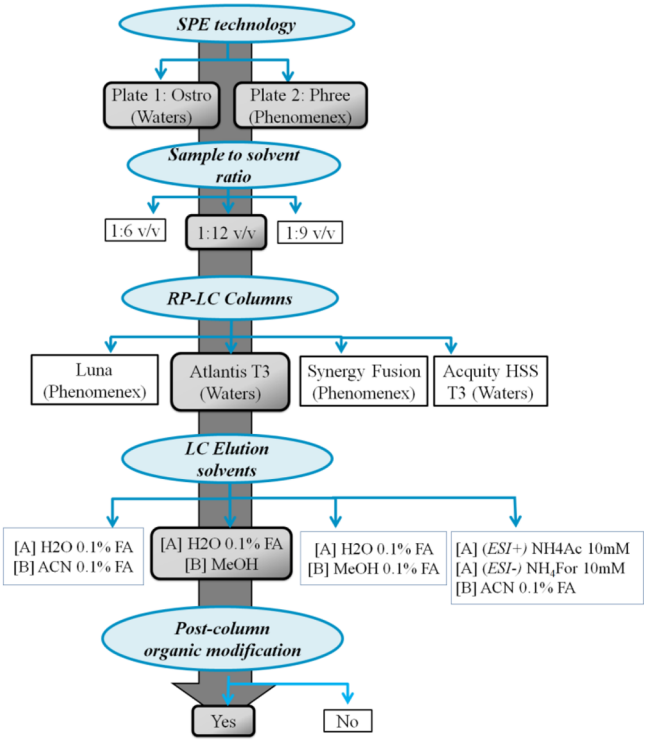

Figure 1. Decision tree-like flowchart showing the methodological scenarios tested in the study.

Samples. Three sample types were used for comparison among the different methodological set-ups: aqueous standard metabolite mixes, reference plasma, and individual human plasma samples collected during a dietary intervention study. An aqueous standard mix (QC2) composed by metabolites representative of the plasma metabolome chemical variety was prepared, including six amino acids, two carnitines, three organic acids, two acyl glycine conjugates, an ester of acetic acid and choline, two fatty acids and two flavonoid compounds (details in Table S-1, Supporting Information). A second standard mix of twenty-one highly polar metabolites (LogP ≪ 0, POL mix) was also prepared for comparison between RP and HILIC chromatographic performance (details in Table S-1, Supporting Information). Aqueous solutions of isotopically labeled compounds were also prepared for use as internal (IS) and external standard (ES) mix during sample extraction (details in the Supporting Information).

Commercial reference plasma (Sigma-Aldrich, St Louis, MO) was used in a first-pass method performance evaluation, to avoid biological variability among compared samples. Plasma was alternatively spiked with milli-Q water (unspiked) and with the QC2 mix (1 and 5 μg/mL final standard concentrations), to evaluate the capacity of the tested methodological set-ups to detect known expected quantitative differences among spiked

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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.

and unspiked reference samples. Human plasma samples from a previously described nutritional intervention study[12,28] were finally used to assess the influence of specific methodological choices in a real metabolomic study (plasma collected before and 2-h after an acute intake of a cocoa-based drink). The choice of the case study was not casual, but pushed by criteria of logic functionality, to easily orient the evaluation of the different methodological options according to the detection of expected biomarkers of cocoa intake.

Sample Extraction Performance Evaluation. *96-well Plate SPE Technologies.* Plasma (50 μL) was subjected to an in-plate hybrid extraction method consisting of deproteinization by acidic solvent precipitation (ACN 1% FA) followed by phospholipid SPE-mediated removal.[12] Two 96-well plates were independently used for sample SPE (plate 1: Ostro, Waters; plate 2: Phree, Phenomenex) according to the respective manufacturer's suggestions.

Sample cleanup from matrix effects was first evaluated by comparing the residual total phospholipid profile of plasma samples extracted through the two 96-well plates, compared to samples subjected to traditional deproteinization techniques alone (organic extraction with ACN, MeOH, MeOH:EtOH 1:1 v/v).[12] This was carried out by monitoring the XIC at m/z 184.070−184.075 (ESI+ mode), corresponding to the yield of trimethylammonium-ethyl phosphate cations released from the residual (lyso)phospholipids still present in the extracts, when applying high-energy declustering potential for in-source fragmentation (+90 V).

Experiments of permanent postcolumn infusion of QC2 standard metabolites were then used. Compared to more conventional approaches (i.e., individual standards addition), the postcolumn infusion technique is in fact a more suitable tool for the evaluation of matrix effects in untargeted LC-MS protocols, because it gives information on signal suppression/enhancement phenomena occurring throughout the whole chromatogram, independently of a specific retention time[29,30] (details in the Supporting Information).

The recovery of QC2 standard metabolites spiked in plasma, the capacity to detect subtle quantitative differences in reference plasma (raw versus spiked) and metabolomic changes in human plasma following an acute cocoa intake were also used to compare the sample extraction performance of the two plates.

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To check for extraction reproducibility, repeated independent extractions (≥3 technical replicates) of each biological sample were carried out. In all cases, aqueous isotopically labeled standard mix were added to the sample matrices before (IS) and after (ES) the extraction (10 μg/mL final standard concentration), to check for extraction reproducibility and analytical stability during LC-MS data adquisition. The successful removal of phospholipids from the samples was also confirmed by including a lysophosphatidylcholine molecule (1-O-stearoyl-*sn*-glycero-3-phosphocholine) in the IS mix (negative control).

Sample-to-Solvent Dilution Ratio (v/v). Three sample-to-solvent dilution ratios (1:6, 1:9 and 1:12 v/v) were then evaluated during the extraction process, according to the plate manufacturer suggestions. For this experiment, reference plasma samples (raw, spiked) were used, and the three dilution ratios were evaluated in terms of the extraction efficiency of the standard metabolites spiked in plasma (peak detection, peak intensities, peak intensity changes among sample classes, $p < 0.05$), the extraction reproducibility (CV of peak intensities among technical replicates) and the shape of the early eluting peaks according to the organic percentage of the extracts.

Chromatographic and Electrospray Ionization Performance Evaluation. *LC Columns, Elution Solvents and Postcolumn Organic Modification.* In line with the choice of maintaining a single-step analysis, four RP column formats with different silica chemistries were first compared (details in "Columns", Supporting Information), ranging from a traditional HPLC C18-based stationary phase to three (U)HPLC columns designed for superior retention of highly polar ionic species. Column performance was evaluated upon a no. ≥450 randomized sample injections, by comparing the retention capacity (mean RT and *k* factor of QC2 standard metabolites showing a wide range of partition coefficients, log *P* −3.19 − +8.23) and the technical reproducibility of the analysis (column back pressure, across-run pressure stability, RT reproducibility by retention time CV).

For ESI enhancement purposes, four mobile phase combinations were then tested (ESI+ and ESI−), differing in the nature of the aqueous [A] and organic [B] phases and in the solvent quality (LC-MS versus ultrahigh performance (UHP)LC-MS purity grade) (details in "Mobile Phases", Supporting Information). Second, the impact of a postcolumn organic enrichment of the mobile phase was also tested by adding a 100 μL/min MeOH flow at the LC-MS interface, so as to reduce the aqueous proportion reaching the MS detector and enhance ESI efficiency (15% increase of organic final concentration). Metabolite detection (peak signal intensities), peak shape, width and symmetry, and the extent of real-life plasma metabolome changes detected in the metabolomics case study were used for intermethod comparison of ESI performance. The performance of the most successful RP-LC-MS setup was finally compared to the use of a HILIC column (XBridge BEH Amide column 100 × 2.1 mm, 4 μm, Waters) in terms of retention capacity and reproducibility, and MS detection sensitivity (POL mix).

The not-mentioned operating conditions for LC-ESI-qToF-MS data acquisition were set as previously described[12] (Supporting Information). To avoid possible bias in intermethod comparison, all extracts resulting from different sample preparation were analyzed in a unique batch-designed and randomized run sequence order, except when not feasible (i.e., comparison among different RP columns, analysis with or without the postcolumn organic modifier). Samples were subdivided into homogeneous sub-batches (~10 injections each) separated by the regular analysis of QC samples (~30% of the total runs). For system suitability check, the following quality control (QC) samples were analyzed throughout the data acquisition: QC1, Milli-Q water samples; QC2 (5 μg/mL final standard concentration); QC3, randomly selected biosamples repeatedly injected along the sequence of analysis. Blank extractions (solvent only) were also injected at the beginning of the sequence, to verify any eventual solvent-dependent mass features not to be considered during comparative analysis. Prior to analysis, a minimum of two QC2 and ten biological samples were injected, respectively to check for system suitability and for system conditioning with the sample matrix.

Analyst QS 2.0 software was used for data acquisition and system control (Applied Biosystems, Foster City, CA, USA).

Chemometric Data Analysis. Before statistical analysis, data preprocessing was carried out using MarkerView 1.2.1. software (AB Sciex, Toronto, Ontario, Canada). Data were $\log_{10}$ transformed and Pareto-scaled to approach a normal distribution, and data quality assured as previously described.[12] Data reproducibility was a common requirement for all the conditions tested.[31] The analytical variability across the runs was then evaluated by monitoring the standard metabolite components of QC2 samples injected over time. Since several of the tested parameters may modify LC and MS response of the QC2 metabolites (i.e., RT shifts and variation in mass signal intensities depending on the different columns, mobile phases and organic modifier), the entire analysis sequence was divided into smaller unmatchable sequences of experiments, and data quality assurance was carried out in intraexperiment separate evaluations (details in "System suitability check", Supporting Information).

Subsequently, UVA (*t* test for pairwise sample comparisons, $p < 0.05$) and MVA (PCA, PCA-DA, OPLS-DA) were applied to the different data sets for comparative analysis, as previously described,[12,32] by using both commercial (MarkerView TM 1.2.1., AB Sciex, Toronto, Ontario, Canada; SIMCA P+ v13, Umetrics, Umeå, Sweden) and online tools (XCMS).[33] PCA score plots, S- and SUS-plots, box plots and relational diagrams were helpful for data comparison and visualization.[34] Finally, the in-house R-based MAIT package[35] was used for the computationally assisted identification of significant metabolites up- or down-regulated in the nutrimetabolomic case study.

RESULTS AND DISCUSSION

Table 1 shows an overview ranking of the methodological options tested, according to the proposed evaluation criteria.

Sample Extraction Performance Evaluation. *96-well Plate SPE Technologies.* The first three criteria used to compare the performance of the two SPE plates were not able to show a clear-cut difference between the sample extraction technologies. In fact, a similar sample cleanup was observed, with negligible levels of residual phospholipids compared to extracts obtained by sample protein precipitation alone (Figure S-2, Supporting Information), resulting in low signal suppression phenomena at the phospholipid elution zone (5.8−8.5 min of the chromatographic run). Similar infusion profiles were also observed, with minimal matrix effects (Figure S-3, Supporting Information). Two suppression zones in the chromatogram (i.e., negative fluctuations in the matrix profiles at ~0−0.5 and 6−7 min) were more accentuated in extracts obtained by the Ostro plate, possibly due to less efficient removal of salts (front solvent) and late-eluting components from the matrix, respectively. However, none of the mass features that were differentiating the extracts in those specific areas corresponded to metabolites of the infused

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QC2 mix, and were considered as artifactual features (signals from chemical impurities, background noise).

Considering the standards recovery (QC2 metabolites spiked in reference plasma), the use of the Phree plate appeared to be associated with an overall enhanced extraction efficiency (percentage recovery in Table S-2, Supporting Information). However, recoveries varied more depending on the analyte monitored than on the SPE technology used, so not to lead to conclusive results (Figure S-4, Supporting Information). Univariate analysis was then used to compare the two extraction technologies upon the capacity to detect subtle quantitative variation in the plasma metabolome ($\lesssim$5 μg/mL-scale changes). In this case, the use of the Ostro plate revealed an increased capacity to detect statistically significant differences between raw versus spiked reference plasma (t test results in Table S-2, Supporting Information), suggesting that an apparent reduced metabolite recovery may not necessarily be associated with a loss of biologically relevant information. Multivariate analysis of the data confirmed the presence of relevant differences among sample extracts, according to the extraction procedure. Figure 2

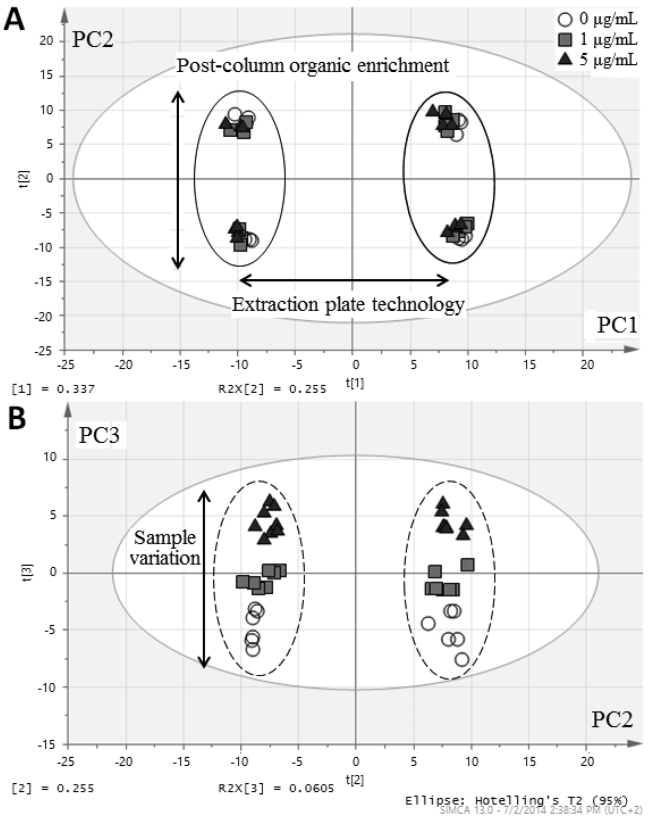

Figure 2. PCA scores plots of plasma samples subjected to extraction by plate 1 versus 2 (discriminated by PC1, solid line circles) and analyzed with or without postcolumn organic modification (discriminated by PC2, dashed line circles); ESI+ mode. Sample classes (plasma unspiked (0) and spiked with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

shows a PCA scores plot of raw versus spiked reference plasma, extracted by the SPE plate 1 versus 2, and analyzed in presence versus absence of the postcolumn mobile phase modification (ESI+ mode). Because the first two PCs of the PCA scores space give the direction of the maximum spread of the data, the exploratory analysis clearly showed how the extraction plate and the postcolumn organic modification of the mobile phase were much stronger determinants of sample variation than the differences among sample classes (similar results in ESI− mode, Figure S-5, Supporting Information).

To obtain a definitive comparison, the nutritional case study was used to assess the extent of metabolomic changes detected in real plasma, according to the sample extraction technology used during sample preparation (supervised multivariate analysis). No difference was observed in the number of significant mass features of cocoa intake between the two extraction plates, with a high extent of data overlapping (Figure S-6, Supporting Information). The most common metabolites expected to be up- and down-regulated following the acute intake of cocoa (i.e., theobromine, caffeine, decanoylcarnitine) were detected in both types of plasma extracts, and confirmed our previous observations.[12,27] However, more robust (O)PLS-DA models were obtained by submitting samples to extraction with the Ostro plate (p-value, R and Q intercepts in Table S-3, Supporting Information). For these reasons, and because the final goal of the application of any metabolomic method is to highlight even subtle metabolite up- and down-regulation in comparative analysis, the Ostro plate finally gave the greatest extraction performance (Table 1).

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

For all these reasons, the use of the highest sample dilution factor was considered the best compromise between extractive capacity, sample dilution and final organic percentage in the extracts (Table 1).

Chromatographic and Electrospray Ionization Performance Evaluation. *RP Columns.* The use of the Atlantis T3 column provided the best chromatographic performance in terms of superior retention of polar metabolites (2-fold higher k factors than by using the traditional C18-based column, for compounds with log P < 0) with negligible effects on the elution of the most nonpolar species monitored and so on the global analysis speed. The column also showed good retention time reproducibility (RT variation < 5 s) (Table S-5, Supporting Information), lower column back pressure and higher across-run pressure stability (pressure variation <5% across up to 800 injections) (Table S-6, Supporting Information). In comparison to the use of a hydrophilic interaction chromatography (HILIC) column (BEH Amide, Waters), the retention capacity and resolution power of polar compounds with structural isomerism

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(i.e., glucose and fructose-6-phosphate) was low ($k < 1$), consistent with the RP nature of the stationary phase. However, peak detection enhancement obtained by HILIC chromatography was neither directly related to the increased retention capacity nor common to all the polar species monitored (i.e., higher sensitivity for D-fructose 1,6-bisphosphate, citric, L-lactic, oxalic, maleic, pyruvic and propionic acids by using RP separation, as shown in Table S-5, Supporting Information). Consequently, these findings did not support a clear-cut improvement by the use of fractionation analysis (RP/HILIC), and confirmed that many factors other than column chemistry can be modulated in order to enhance sensitivity in MS.

Elution Solvents. Although the effect on signal response was found to be compound dependent, the use of nonacidified MeOH as organic phase [B] gave the strongest molecular ion signal intensities, in both ionization modes (Figure S-8, Supporting Information). Particularly, in ESI− mode, the use of MeOH increased peak intensities up to 25-fold compared to the reference organic phase used in previously validated protocols (ACN 0.1% FA).[32] Moreover, peak broadening effects putatively expected with the use of MeOH[36] were negligible in respect of the gain in peak intensity enhancement observed (Figure S-8, Supporting Information), and were neither shared by all the monitored metabolites nor detrimental for peak detection during data preprocessing.

Finally, the use of UHPLC-MS quality grade MeOH gave between a 2- and 37-fold increase in peak intensities, compared to the ACN-based organic phase (Figure S-8, Supporting Information, ESI− mode).

Postcolumn Mobile Phase Modification. The organic enrichment of the mobile phase prior to the MS detection was a strong determinant of sample variation (Figure 2). In fact, it consistently associated with a significant enhancement of mass signal intensities for all the monitored metabolites eluting up to two-thirds of the chromatogram (Figure 3), leading to an increased capacity to detect up to 1 μg/mL-scale changes between raw and spiked reference plasma samples. A decrease in mass signal intensities was not common among the late-eluting metabolites, and loss of peak detection did not occur at the concentration range monitored (1−5 μg/mL), suggesting that the diluting effect of the extra volume of mobile phase infused did not dramatically affect metabolite detection (dilution effect). Furthermore, peak broadening (putatively expected due to dead volume introduction) was again negligible and not associated with the loss of peak detection. Finally, the postcolumn organic modification of the mobile phase also enhanced the detection of significant biological changes occurring in the plasma metabolome after an acute dietary intervention.

To verify the impact of using this analytical solution in the enhancement of ESI efficiency and the detection of subtle metabolomic differences among real sample classes, the number of biomarkers detected in plasma following an acute intake of cocoa, in the absence or presence of the postcolumn modification of the mobile phase, was compared. Figure 4 shows a SUS-plot comparing the significant mass features detected by the two models (model 1, with mobile phase modification; model 2, without mobile phase modification). As shown in the figure, although the strongest biomarkers of cocoa intake were detected independently on the use of postcolumn addition (mass features found on the plot diagonal represent shared biomarkers, i.e., theobromine and caffeine), other previously described metabolites were uniquely detected through the organic modification of the mobile phase (mass features far from the diagonal, i.e. up-regulation of glycochenodeoxycholic acid, down-regulation of L-(iso)leucine).[12,37] Nevertheless, the majority of the cocoa-associated discriminant mass features observed following

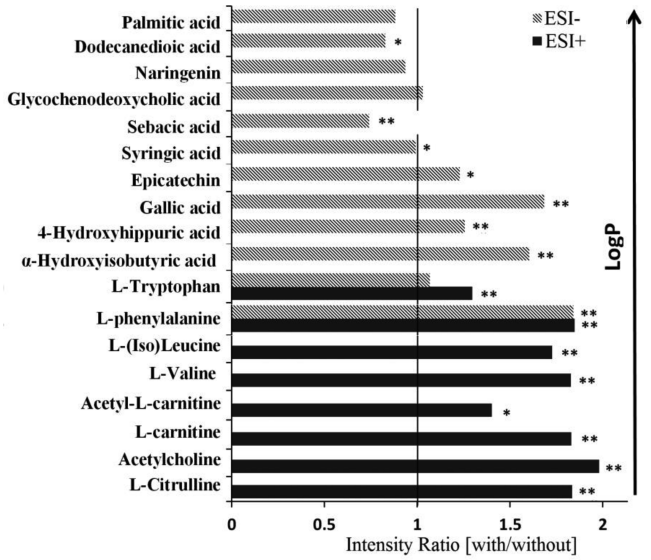

Figure 3. Ratios of mass signal intensities obtained with vs without the use of postcolumn modification of the mobile phase (*, significant differences, $p < 0.05$; **, highly significant differences, $p < 0.01$). Intensities were expressed as mean of five technical replicates. CV ranged from 1 to 21%.

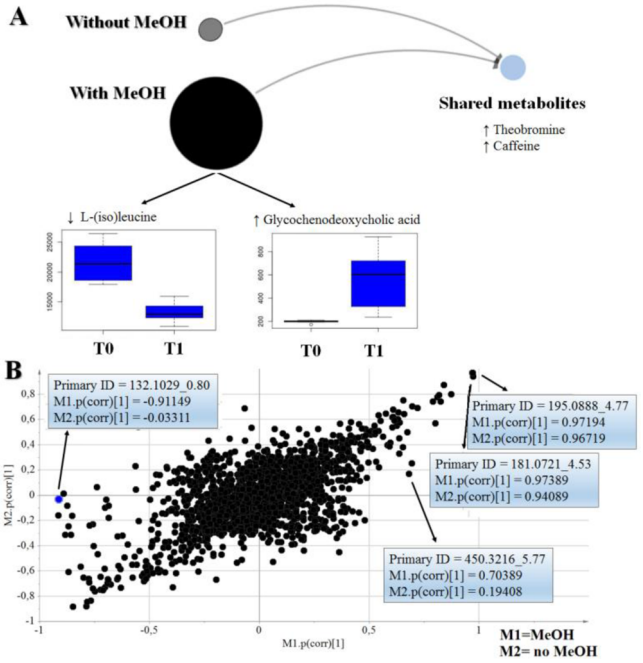

Figure 4. (A) Relational diagram matching the extent of metabolomic changes detected in plasma after an acute dietary intervention by using or not the postcolumn organic modification of the mobile phase (metaXCMS). Uniquely identified metabolites significantly down-(left) and up-regulated (right) following 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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postcolumn organic modification kept unidentified, hampering to fully evaluate the extent of method performance enhancement.

CONCLUSION

Although being excellent samples for assessing pathophysiological deviations in both endogenous and exogenous metabolism, blood fluids are as informative as challenging samples.[38] Inadequate preparation and LC-MS analysis of these complex biomatrices are major sources of ion suppression phenomena, which in turn adversely affect the most crucial prerequisite for untargeted metabolomics, such as metabolome coverage, mass accuracy, and data reproducibility. However, there is still no consensus on how to overcome specific analytical hindrances and make large-scale untargeted studies feasible. Even the method performance evaluation process is not fully standardized, due to the difficulty in defining validation criteria to cover the specific problems associated with untargeted analysis of (mostly unknown) compounds. Although the concept of method optimization is not viable in untargeted metabolomics, the joint finishing of sample extraction procedure, chromatographic separation and electrospray ionization still forms the necessary basis to develop a successful and robust LC-ESI-qToF-MS (qToF, quadrupole time-of-flight) methodology, and reach a balanced compromise between metabolome coverage and feasibility.

In the present work, we investigated the response of the plasma metabolome coverage to specific methodological choices related to sample preparation, chromatographic separation and ESI process enhancement procedures in RP-LC-MS data adquisition.

The best sample extraction performance was obtained when combining sample hybrid extraction with high organic proportion. During sample preparation, both a partial removal of undesired interference or a too drastic nonselective cleanup of the samples would lead to poor metabolome coverage and compromise the detection of subtle metabolite variation in comparative analysis.[8,19−21] In a previous work, we observed that the selective removal of phospholipid-based matrix effects is a more successful alternative for plasma sample preparation than deproteinization alone[12] (the "less is more" concept). In the present work, two in-plate hybrid extraction technologies were compared, and conventional criteria for extraction efficiency evaluation were not definitive in making lean toward one or the other plasma preparation procedure. Nevertheless, the observed findings suggested that an apparent reduced recovery of specific standard metabolites is not necessarily associated with a loss of biologically relevant information, confirming that established criteria for the optimization and validation of targeted LC-MS/MS bioanalysis should be integrated with untargeted approaches for method performance evaluation, in order to get closer to the real objectives of the analytical protocols.

Although no solvent removal/replacement was carried out after sample extraction and prior to LC-MS analysis, neither significant loss of metabolite detection nor early eluting peak shape distortion were observed in our study, suggesting that extract evaporation and reconstitution steps may not be truly necessary following the proposed sample preparation protocol. Extract evaporation followed by reconstitution is commonly incorporated in sample preparation procedures, generally justified by solvent removal/replacement and low-sensitivity issues (concentration purposes). However, concerns about introducing these potentially "risky" steps are widely shared (risk of incomplete solubilization of the dry residues and losses of hydrophobic and volatile species, increased ion suppression due to preconcentration of interferents, reduced sample throughput due to considerable time consumption), and no evidence has been produced so far of the actual benefits in terms of metabolome coverage.[22] Furthermore, the sample-to-solvent ratio giving the highest final plasma dilution and greatest organic proportion was associated with a higher extractability of the monitored metabolites, without compromising peak detection and shape. The tested ratios were chosen upon the plate manufacturer suggestions and consistently with the challenge of minimizing ion suppression phenomena while bypassing subsequent sample concentration. Although the best dilution ratio is not an absolute parameter to extrapolate for application to different extraction procedures, our findings suggested that sample dilution may be a valuable choice to reduce matrix effects and enhance metabolome coverage.

The use of Atlantis T3 RP column compatible with 100% initial aqueous phase was then found the best option to maximize retention of polar metabolites. The replacement of acidified ACN with nonacidified MeOH as organic phase [B] gave the strongest molecular ion signal intensities, in both ionization modes. Although acidification of both mobile phases of the elution gradient is a common technique in LC-UV analysis, in order to avoid major baseline disruptions when working at low-absorbance wavelengths (i.e., 210 nm), this rule does not apply in LC-MS data acquisition, allowing the best binary systems to be chosen in view of the ionization enhancement. Besides showing the best spray solvent characteristics in terms of volatility and surface tension (low dielectric constant and viscosity), MeOH is also known to facilitate lipid elution and avoid accumulation in the column (i.e., as acetone), which may further contribute to the minimization of matrix effects.[27] Furthermore, compared to harmful solvents such as ACN, MeOH is a more "environmentally friendly" alternative in terms of workers, processes and environment safety (i.e., ecotoxicity),[39] and its replacement in LC applications should be encouraged as a "greener" option.[40,41] The purity grade of the solvents used in mobile phase preparation revealed to be an even more crucial factor in mass signal detection, although at least LC-MS purity grade solvents were used. These findings underlined the importance of checking the levels of contaminants in the solvent, such as plasticizers and surfactants, which are very often readily ionizable and can compete for charge with the metabolites.[42]

Finally, the postcolumn modification of the mobile phase by organic enrichment confirmed to be a pragmatic solution to increase the ionization efficiency without influencing the chromatographic separation, although no applications in RP-LC-ESI-MS untargeted metabolomics have been described until now. Other postcolumn solvents apart from methanol were not considered in our flowchart, for several reasons. The use of the same solvent for both mobile phase and postcolumn enrichment of the phase has been suggested as preferable, and the use of nonacidified MeOH as organic phase [B] gave the strongest molecular ion signal intensities in both ESI modes. Both MeOH and ACN are considered as adequate as postchromatography organic modifiers, but they do not differ strongly in decreasing the droplet surface tension in the ESI source, and so in helping ionic evaporation. However, MeOH shows some peculiar advantages in respect to acetonitrile, such as a higher vapor pressure and a lower surface tension, which facilitate even more solvent evaporation.

Summarizing, the results of the present work led to the following considerations: plasma samples should be submitted to

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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

Supporting Information

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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Notes

The authors declare no competing financial interest.

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