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Title: APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE

Article Type: Research Paper

Keywords: Alzheimer's disease; Metabolomics; Atmospheric pressure photoionization; Flow injection

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Abstract: The use of atmospheric pressure photoionization is not widespread in metabolomics, despite its considerable potential for the simultaneous analysis of compounds with diverse polarities. This work considers the development of a novel analytical approach based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for rapid metabolic screening of serum samples. Several experimental parameters were optimized, such as type of dopant, flow injection solvent, and their flows, given that a careful selection of these variables is mandatory for a comprehensive analysis of metabolites. Toluene and methanol were the most suitable dopant and flow injection solvent, respectively. Moreover, analysis in negative mode required higher solvent and dopant flows (100 µl min-1 and 40 µl min-1, respectively) compared to positive mode (50 µl min-1 and 20 µl min-1). Then, the optimized approach was used to elucidate metabolic alterations associated with Alzheimer's disease. Thereby, results confirm the increase of diacylglycerols, ceramides, ceramide-1-phosphate and free fatty acids, indicating membrane destabilization processes, and reduction of fatty acid amides and several neurotransmitters related to impairments in neuronal transmission, among others. Therefore, it could be concluded that this metabolomic tool presents a great potential for analysis of biological samples, considering its high-throughput screening capability, fast analysis and comprehensive metabolite coverage.

Manuscript TAL-S-14-01551

Reviewers' comments

Study design: 30 AD and 30 controls were selected. However no medical information were provided thus it is hard to judge the observed phenomenon is due to AD or drug effect. Please add a table describing the patients information with especially the MMSE, CDR or other scores, APOE genotype. Explain when the samples were collected (after fasting, morning?) because this may affect the data. Some AD patients may take medicines such as donepezil. Did authors find those drug peaks? If they did not, this may be due to low sensitivity of this method (ion suppression).

A new file is provided as supporting information containing demographic characteristics of subjects enrolled in the study (age, gender, co-morbidities, and medication). Given the advanced age of patients, most of them suffered other co-morbidities and were under different medical treatments, but there were not significant differences among the groups considered, in line with previous works (see PLOS ONE 2013 8:e63644). In addition, it is noteworthy that AD patients included in the study had not yet received any type of medication (e.g. donezepil, memantine), in order to study early markers of disease and avoid confusing metabolic alterations derived from this type of treatment.

In the revised manuscript is specified that serum samples were collected in the morning.

Flow-injection approach is good for high through-put careening, but the limitations are 1) the separations of the same (isomers) or similar m/z products (within 0.1 Da) and 2) ion suppression due to highly complex samples. Authors have to seriously consider these limitations. For instance, authors quantified several metabolites, but they have to show those MS spectra and their theoretical isotope distributions. When they do not match well between experimental and theoretical, those peaks are probably overlapped each other. Please describe the probability of each identified metabolite and how to quantify each peak. Ion suppression is another concern, because authors identified only highly abundant metabolites, especially lipids. Please describe this aspect, and the detection limits of several metabolites in human serum.

The importance of these drawbacks associated with ion suppression and lack of resolution of isomers has been highlighted in the Introduction section. In order to confirm the reliability of the identification of metabolites in relation to the possible overlapping with isobaric compounds, we introduced in Table 4 the most important ions detected in MS/MS spectra of these metabolites. On the other hand, although we can not provide detection limits of metabolites because this is not a quantitative study, a recent assertion from Draper et al. (Metabolomics 2013 9:S4-S29) establish that “it is difficult to find any evidence that ion suppression has more of a detrimental effect on flow infusion fingerprinting than in LC–MS methods using chromatography to separate metabolites”, as recently reviewed by Draper et al. (Metabolomics 2013 9:S4-S29). In this sense, Boernsen et al comment that “in general, the large amount of different compounds within plasma helps stabilize the complete system and regulate suppression effects” (Anal Chem 2005 77:7255-7264). Therefore, it is presumable that ion suppression observed with this technique is similar to that reported with other metabolomic platforms.

It is also not convincing to me that only using FIA and QSTAR, the authors could accurately identify the listed metabolites in complex sample like serum. In metabolomics, even using LC separation coupled with high resolution accurate mass with less than 5ppm, as well as MS2 fragment information, it is still challenging to do metabolite identification.

Please describe how to calibrate their mass spectrometer and how often do they calibrate QSTAR XL. To keep 50 ppm accuracy, QSTAR needs frequent calibration or internal/retrospective calibration.

For accurate mass measurement, the TOF analyzer is calibrated before analyzing each batch of samples (30 AD + 30 HC) using renin as standard. Given the rapidity of analysis (less than 1 minute per sample), the instrumental drift along the analysis period is considerably reduced, which allows to keep an appropriate accuracy.

The authors mentioned that they compared the tandem mass spectra (MS/MS) with those from HMDB; however in the INSTRUMENTATION section, there is no description about how they set up the information dependent acquisition (IDA) experiment in the QSTAR to collect tandem mass spectra.

A new sentence was introduced in the Instrumentation section describing how is performed the acquisition of MSMS spectra.

PLS-DA score plot (figure 6) showed the separation between male and female subjects. There is nothing related to AD and control separation.
Figure 6 shows the score plot for the separation between AD patients and healthy controls (not female vs male). This error has been corrected.

Q2 is a parameter in PLS-DA for cross validation and permutation test. The values in table 3, which are close to 1, indicate that the models are overfitting. Metabolites selected for AD from these PLS-DA's VIP values, to me, are not reliable.
PLS-DA models were validated using permutation tests. Permuted models showed lower R2 and Q2 values than original models, indicating that models are not overfitted. This has been mentioned in the revised manuscript.

In general, Arg (basic aminoacid) would show stronger intensity in positive mode rather than acidic aminoacids (Glu, Asp), and Glu/Asp would be stronger than His in negative mode. Their data described in fig 1 and fig 2 is opposite. Please describe the reasons.
This rationale is valid in electrospray, where acid/basic properties play a prominent role in ionization. However, compounds can be ionized through different mechanisms in APPI (proton transfer and charge exchange reactions), so a different trend can be observed in relative ion intensities with regards to ESI.

Are there any reasons why authors did not go down the flow-rate from 50 uL/min in positive mode? It seems to be more sensitive at lower flow-rate.
50 uL/min is the minimum flow rate allowed in the LC system employed (Accela 600) with an optimal performance.

When authors add enough amount of dopant, is "100 uL/min" still optimal flow-rate in negative mode?
Yes, we confirm that a higher solvent flow is needed in negative mode after optimizing the flow of dopant.

How many metabolites did authors identify in this system? Why can they say APPI is superior to ESI/APCI? Authors need comparison data or solid evidences.
The purpose of this study was the development of a complementary (not superior) approach for metabolomic analysis of serum samples given that, as stated in the Introduction, due to the high complexity of the metabolome there is a growing need to develop multiple platforms to get a comprehensive assessment of metabolites with very diverse chemical and physical properties.

Authors should mention reproducibility of this method (CV values). It's hard to consider the meanings of 1.1-fold increase without recovery/reproducibility data.
CV values of metabolites identified have been listed in Table 4 (calculated from a sample analyzed in triplicate).

Infusion analysis requires multiple internal standards to improve reproducibility. Authors should mention this point.
We did not use internal standards to improve analytical reproducibility because the instrumental drift in ion intensities between measurements was corrected by statistical normalization according to the total area sum (see Statistical Analysis section), as previously described in other works based on direct infusion ESI-MS (J Proteomics 2014 104:37). Furthermore, the lack of a chromatographic step before MS detection avoids the need of standards for the alignment of retention times.

Table 4 describes only "Fold change". Please add biostatic data.
P-values have been included in this table.

Table4 shows some metabolites are identified in both positive and negative modes, but the "fold change" value is only one, which means positive and negative modes provide the exact same values?
These values are similar, but not exactly the same. The fold changes presented in Table 4 are mean values.

Please provide some MS/MS data with chemical structures, especially for isomers.
As stated in the response to the second question of your comments, we introduced the most important ions detected in MS/MS spectra of these metabolites in Table 4.

Please create ROC graph with sensitivity and specificity (this is more common than what they describe)
ROC analysis was performed, and AUC values for potential markers identified in this study have been appended in Table 4.

As future plan, please look at their metabolites in CSF and if possible in post-mortem AD brains. At least they should mention their next step in the conclusion.
In this case, they measured only AD samples, but please look at other dementia types and MCI: now the separation is so good that it seems to me that this is not an AD markers but general markers of neurodegeneration. From our trials, 50% that are classified based on the test they used turned out to be amyloid negative (AV-45). At least mention this in conclusion part as their next step
We have taken into account these considerations in the Conclusions section.

The introduction should explain even briefly what is Alzheimer's Disease.
A brief explanation about Alzheimer's disease has been added to the Introduction section.

***Highlights (for review)**

APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE

Raúl González-Domínguez, Tamara García-Barrera, José Luis Gómez-Ariza

- FIA-APPI-MS provides comprehensive metabolomic fingerprints of serum samples
- This approach allowed the study of metabolic alterations in Alzheimer's disease
- Important failures were detected in membrane lipids, neurotransmitters and others

*Graphical Abstract (for review)

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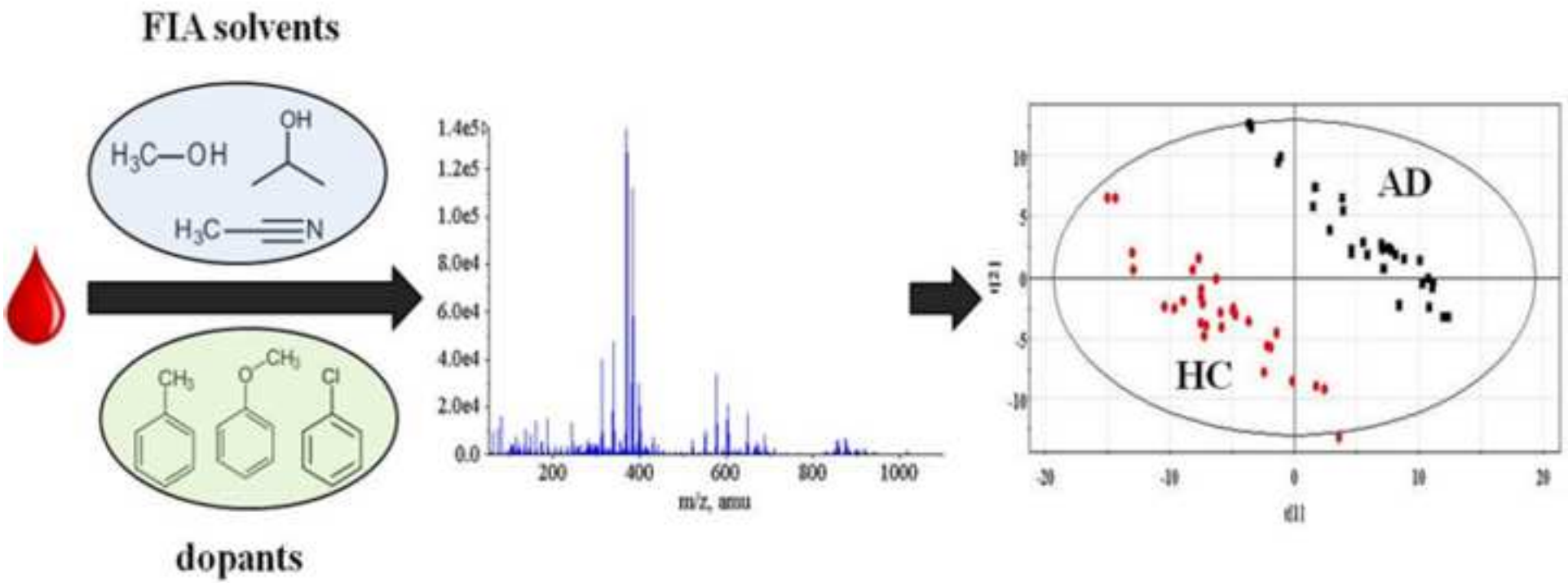

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The use of atmospheric pressure photoionization is not widespread in metabolomics, despite its considerable potential for the simultaneous analysis of compounds with diverse polarities. This work considers the development of a novel analytical approach based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for rapid metabolic screening of serum samples. Several experimental parameters were optimized, such as type of dopant, flow injection solvent, and their flows, given that a careful selection of these variables is mandatory for a comprehensive analysis of metabolites. Toluene and methanol were the most suitable dopant and flow injection solvent, respectively. Moreover, analysis in negative mode required higher solvent and dopant flows (100 µl min^{-1} and 40 µl min^{-1}, respectively) compared to positive mode (50 µl min^{-1} and 20 µl min^{-1}). Then, the optimized approach was used to elucidate metabolic alterations associated with Alzheimer's disease. Thereby, results confirm the increase of diacylglycerols, ceramides, ceramide-1-phosphate and free fatty acids, indicating membrane destabilization processes, and reduction of fatty acid amides and several neurotransmitters related to impairments in neuronal transmission, among others. Therefore, it could be concluded that this metabolomic tool presents a great potential for analysis of biological samples, considering its high-throughput screening capability, fast analysis and comprehensive metabolite coverage.

Keywords
Alzheimer's disease, metabolomics, atmospheric pressure photoionization, flow injection.

Novelty statement
This work describes for the first time the use of a metabolomic platform based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for the investigation of metabolic alterations associated with Alzheimer's disease.

INTRODUCTION

The main challenge in metabolomics is to obtain comprehensive and unbiased metabolic fingerprints of samples due to the huge heterogeneity and dynamism of metabolome [1]. In this context, mass spectrometry represents a very interesting analytical platform, since complexity of metabolome may be overcome through the use of complementary atmospheric pressure ionization methods [2]. Electrospray (ESI) is the most common ionization source employed in metabolomic studies because it is able to ionize compounds in a wide range of masses and polarities, and it may be coupled to liquid chromatography [3-4], capillary electrophoresis [5-6] or used by direct infusion mass spectrometry [7]. A second alternative is atmospheric pressure chemical ionization (APCI), more suitable for less polar compounds [8-9]. Finally, atmospheric pressure photoionization (APPI) complements ESI and APCI for the analysis of little polar or non-polar compounds, but it has been considerably less used in metabolomics. Atmospheric pressure photoionization is the most recent soft ionization technique for mass spectrometry, introduced simultaneously by Bruins and Syage in 2000 [10-11], extending the range of ionizable compounds to less polar ones, which are not readily ionized by ESI and APCI. Nevertheless, APPI is capable of ionizing both polar and non-polar compounds through proton transfer and charge exchange reactions respectively, so it could be considered a universal ionization source [12]. The APPI interface uses a photoionization lamp and a dopant flow to form dopant radical ions, which can directly ionize non-polar analytes through charge exchange reactions. On the other hand, for polar compounds dopant photoions can produce intermediate reactive species by reactions with solvent or oxygen molecules, in positive and negative ionization modes respectively, followed by a proton transfer reaction to the analyte [12]. Thereby, for the simultaneous analysis of both polar and non-polar compounds, the two reaction pathways must be accessible, which requires a careful selection of solvent, dopant, and their flows. Firstly, the carrier solvent employed can lead to a preferential mechanism, being favored charge exchange for low proton affinity solvents, whereas the addition of methanol or acetonitrile initiates proton transfer [13]. In the case of dopant, the most common reagent is toluene, but it may be not suitable for the ionization of non polar compounds in high proton affinity solvents since tends to transfer its proton to the solvent [14]. Alternatively, other dopants less reactive with the solvent have been proposed, such as anisole [15] or substituted benzenes [16]. Finally, the flow-rate of both solvent and dopant also play important roles in the photoionization process. In the case of solvent, ionization efficiency decreases when the flow increases, due to the formation of large unreactive protonated-solvent cluster ions, while signal increases with the dopant flow, until reach 5-10% of solvent flow [17]. Moreover, it has been reported that toluene can provide high ionization efficiency simultaneously to both polar and non-polar compounds delivered in reversed-phase solvents, simply limiting the solvent flow rate in order to avoid that reactions between toluene photoions and solvent were driven to completion [18].

In this way, APPI might present a considerable potential in metabolomics, not only because of its universality in terms of ionization capability, but also because is less susceptible to matrix effects and presents a linear dynamic range generally higher than that for ESI [19-20]. In addition, it requires less heat for desolvation than APCI allowing the analysis of thermally labile compounds [21]. APPI has been used for the analysis of many classes of compounds, including pharmaceutical drugs and metabolites [22], steroids [21], aldehydes and ketones [23] or pesticides [24]. However, only a few non targeted approaches include APPI as an alternative in metabolomics. Thereby, metabolomics based on liquid chromatography mass spectrometry and the integration of multiple ionization modes (ESI, APCI and APPI) has been previously proposed for the study of urine [25] and plasma samples [26], or to perform a more comprehensive analysis of lipidome [27-28]. Although APPI is normally coupled to liquid chromatography [29] or capillary electrophoresis [30], several reports confirm the ability of flow injection analysis for the determination of fullerenes and perfluorinated compounds [31], characterization of wine [32], olive oil [33] or Iberian ham [34], or the study of drugs [35] and petroleum [36]. This high-throughput approach exhibits several advantages in metabolomics such as fast and reproducible analysis, comprehensive metabolite coverage and simple data pre-processing [7], but it also presents important drawbacks associated with the lack of resolution for the differentiation of isobars and difficulty of quantification due to ion suppression. In order to overcome problems associated with isobaric interferences, the use of high resolution systems has become the main workhorse for accurate MS-fingerprinting, including time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR), and especially the hybrid system Q-TOF, which allows more accurate mass measurement than single TOF instruments and structural elucidation by MS/MS experiments [37]. On the other hand, although ion suppression is a potential problem in any MS-based metabolomic platform, there is no evidence that it presents a more detrimental effect on flow infusion fingerprinting than in hyphenated approaches [7]. In fact, the large amount of different compounds in biological samples helps to regulate matrix effects, so that ion suppression becomes a constant factor imposed uniformly in all samples derived from similar types of tissues [38]. Thereby, MS-fingerprinting has proved to be an excellent tool for high-throughput metabolomic characterization of complex samples, usually using electrospray

ionization, as recently reviewed by Draper et al. [7]. However, to date, the use of FIA-APPI-MS has not been considered in metabolomic analysis.

This work explores for the first time the potential of flow injection analysis and high resolution tandem mass spectrometry with atmospheric pressure photoionization source (FIA-APPI-QTOFMS) in metabolomics. Several critical parameters were optimized for the simultaneous analysis of both polar and non-polar compounds in positive and negative ionization modes, such as type of dopant, flow injection solvent and their working flows. For this purpose, a test mixture of representative metabolites from human serum was used. Then, the optimized approach was applied to blood serum samples in order to investigate metabolic abnormalities associated with Alzheimer's disease (AD). This neurodegenerative disorder is poorly understood, and its etiology is still unknown, although it is likely to be a conglomeration of different pathological entities. There is currently no cure for Alzheimer's disease, but early diagnosis could help monitor disease progression and target therapies earlier in the course of the disease, so identification of reliable biomarkers is becoming increasingly important. Multivariate statistics demonstrated the ability of this high-throughput metabolomic tool for discriminating between AD patients and healthy controls, and allowed the identification of different metabolic failures underlying to pathological features of this health disorder.

MATERIAL AND METHODS

CHEMICALS AND SAMPLES

The solvents (HPLC-grade) methanol, ethanol, chloroform, dichlorometane, acetonitrile and isopropanol were purchased from Fisher Scientific (Leicestershire, UK), while dopants toluene, anisole and chlorobenzene were supplied by Aldrich (Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Standards of L-glutamine, L-valine, L-cysteine, L-aspartic acid, L-arginine, L-histidine, L-glutamic acid, L-phenylalanine, D-glucose, creatine, creatinine, cholesterol, di-oleoyl-phosphocholine and triolein were from Sigma Aldrich. Blood samples were collected in the morning by puncture of the antecubital vein and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. The samples were immediately cooled and protected from light for 30 minutes, and after centrifugation (3500 rpm for 10 minutes) serum was aliquoted and frozen at -80°C until analysis. Alzheimer's disease patients (AD, N=30, age 80.3±5.0, male/female 12/18) were newly diagnosed of sporadic Alzheimer's disease by the Neurologic Service of Hospital Juan Ramón Jiménez (Huelva, Spain), according to the criteria of the NINCDS-ADRDA [39], and only subjects that had not yet received any type of medication were included in the study. Healthy controls (HC, N=30, age 73.5±5.9, male/female 10/20), who had not more than two reported cases of Alzheimer's disease in their families, were studied by neurologists to confirm the absence of neurological disorders. Demographic characteristics of groups considered in the study are listed in the Supporting Information, including age, gender, co-morbidities, medication and family history of AD. It is noteworthy that most subjects suffered other co-morbidities and were under different medical treatments, but there were not significant differences among the groups considered. The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of University of Huelva.

PREPARATION OF SAMPLES

Optimization of experimental conditions was performed with a solution containing standards of representative metabolites from blood serum in methanol:water (1:1, v/v), including different low molecular weight compounds from different chemical classes and lipids, ranging diverse acid-base properties and polarities. The composition of this mixture was selected according to their usual concentrations in human serum described in the Human Metabolome Database (HMDB), as shown in Table 1. This metabolomic approach was further tested in real serum, for which samples were treated in a two-stage sequential procedure described elsewhere for comprehensive serum metabolomics [40]. For the extraction of metabolites, 100 µL of serum are mixed with 400 µL of methanol/ethanol 1:1 and stirred for 5 min, followed by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant is transferred to another tube, and the precipitate is kept for further treatment. Then, supernatant is dried under nitrogen stream and the resulting residue reconstituted with 80 µL of methanol and 20 µL of water (polar extract). On the other hand, the precipitate isolated in the first step is extracted with 400 µL of chloroform:methanol 1:1 by stirring during 5 min, followed by centrifugation at 10000 rpm for 10 minutes at 4 °C. Finally, the resulting supernatant is taken to dryness under nitrogen stream and reconstituted with 100 microliters of 60:40 dichloromethane:methanol (lipophilic extract).

INSTRUMENTATION

Mass spectrometry experiments were performed on a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure photoionization (APPI) source. Samples were introduced by flow injection using an Accela LC system (Thermo Fisher Scientific) equipped with autosampler and quaternary pump. In

addition, a model KDS 100 syringe pump from KD scientific (New Hope, PA, USA) was employed to deliver the dopant for photospray ionization. For accurate mass measurement, the TOF analyzer is calibrated before analyzing each batch of samples using renin as standard. Data were obtained both in positive and negative ion modes, injecting 10 µl of sample, and acquiring full scan spectra in the m/z range 50-1100 with 1.005 seconds of scan time. Acquisition time was selected according to the LC flow employed to consider the dispersion of the plug of sample into the solvent stream, and employing some delay time between injection and analysis to allow enough time for the sample to reach the mass spectrometer (settings shown in Table 2). The ion spray voltage (IS) was set at 1500 V and -2300 V in positive and negative modes respectively, with declustering potential (DP) of ±50 V and focusing potential (FP) of ±250 V. The source temperature was maintained at 400ºC, and the gas flows (high-purity nitrogen) were fixed at 1.13 L min^{-1} for curtain gas, 1.50 L min^{-1} for nebulizer gas, 3.0 L min^{-1} for heater gas, and 1 L min^{-1} for lamp gas. To acquire MS/MS spectra, nitrogen was used as collision gas and the collision energy was ranged between 10-60 V. The optimization of FIA conditions was performed in four steps: type of dopant, type of carrier solvent, solvent flow and, finally, dopant flow. For the analysis of serum samples, the optimal dopant was toluene delivered at 20/40 µl min^{-1}, in positive and negative ion mode respectively. Furthermore, methanol was used as flow injection solvent at 50/100 µl min^{-1}, in both ionization modes.

STATISTICAL ANALYSIS

Metabolomic data were submitted to peak detection by Markerview™ software (Applied Biosystems) in order to filter the mass spectrometry results, and to carry out the reduction into a two-dimensional data matrix of spectral peaks and their intensities. For this, the peak search was done with a mass tolerance of 0.1Da, and a minimum response of 10 counts was considered for filtering. Finally, data were normalized according to the total area sum. Then, data was processed in SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden) in order to find differences between the groups of study (AD patients and healthy controls). Partial least squares discriminant analysis (PLS-DA) was performed to build predictive models that provide class separation and allow the further study of discriminant metabolites. Quality of the models was assessed by the R^2 and Q^2 values, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). In addition, these models were validated using permutation tests (Y-scrambling) of the Y-predicted values. In Y-scrambling, class labels are randomly permuted for refitting a new model with the same number of components as the original one, and then these new models are compared with the original models to test the possibility that the original model arose by chance. Thus, an overfitted model will have similar R^2 and Q^2 to that of the randomly permuted data, while well fitted and meaningful models will have R^2 and Q^2 values higher than that of the permuted data. Finally, discriminant compounds were selected according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 2.0, indicative of significant differences among groups. Furthermore, these potential biomarkers were subjected to receiver operating characteristic (ROC) analysis to assess their diagnostic ability. The ROC curve analysis was performed using the GraphPad Prism software (version 6.04, Intuitive Software for Science, San Diego, CA), and the area under the curve (AUC) was used as a metric of sensitivity and specificity of these biomarkers. Thereby, a marker is excellent when AUC ranges from 0.9 to 1, good if AUC is 0.8 to 0.9, moderate if AUC is 0.8 to 0.7 and poor below 0.7 [41].

IDENTIFICATION OF METABOLITES

Discriminant metabolites were identified matching the experimental accurate mass and tandem mass spectra (MS/MS) with those available in metabolomic databases (HMDB, METLIN, LIPIDMAPS), using a mass accuracy of 50 ppm. Then, different classes of lipids were confirmed based on characteristic fragmentation patterns previously described. It is noteworthy that signals from APPI mass spectra can be mainly attributed to protonated or deprotonated adducts (in positive and negative modes, respectively), but the possibility to produce dehydrated molecular ions has been also previously demonstrated [42-44]. Ceramides presented characteristic product ions in positive ionization mode at m/z 264 and 282 due to the fragmentation in the sphingosine moiety [45]. On the other hand, fragmentation of glycerolipids (diacylglycerols) occurs through the release of fatty acids generating different types of ions (named A, B, C and D), which show characteristic m/z values according to the fatty acid attached to the glycerol backbone [34]. Finally, fatty acid amides were confirmed with characteristic fragments described in the literature [46].

RESULTS AND DISCUSSION

SELECTION OF EXPERIMENTAL CONDITIONS

The effect of experimental conditions on the photoionization efficiency was evaluated by flow injection analysis of a mixture solution of representative metabolites (Table 1), acquiring in both positive and negative ion modes. Firstly, for the selection of dopant the other parameters were adjusted according to previous studies for the simultaneous determination of both polar and non-polar compounds [18], employing a mixture of methanol:water (1:1, v/v) as solvent at a flow rate of 100 µl min^{-1}. Dopant flow was fixed at a 10% of solvent flow (10 µl min^{-1}), since a plateau in ionization efficiency is reached in this source as more dopant is added [17]. The most common dopants described in literature were tested in this study (i.e. toluene, chlorobenzene and anisole), and the ionization efficiencies were compared in function of peak intensities (Fig. 1). In positive ionization mode (Fig. 1a) the best sensitivity was obtained with toluene for most of the standards, followed by chlorobenzene (it should be noted that the great increase in intensity observed for histidine with chlorobenzene is attributable to the presence of an impurity in this solvent with the same mass, which was checked using a blank test). However, for lipid compounds the peak intensity is slightly increased with anisole and chlorobenzene, although none of these dopants allowed the ionization of di-oleoyl-phosphocholine. On the other hand, intensities of low molecular weight metabolites were very similar in negative mode using the three dopants, although generally lower than that in positive mode, while lipids were not detected. The enhanced ionization observed with toluene and chlorobenzene compared to anisole could be due to their higher ionization energy (IE) values (8.83, 9.07, and 8.20 eV, respectively), given that for an effective photoionization the IE of the dopant must be higher than that of the analytes. Moreover, the better sensitivity of toluene compared to chlorobenzene for polar metabolites in positive mode, despite its lower IE, may be due to the impossibility of chlorobenzene to perform photoinozation by proton transfer [16]. Conversely, this low reactivity of chlorobenzene and anisole with the solvent is also responsible for the increased signal of non-polar analytes such as cholesterol and triolein using these dopants, although good results were also obtained with toluene. Therefore, toluene was selected as dopant for further experiences, since it provides acceptable results with most of metabolites with the highest mean sensitivity. In a second step, different solvents were evaluated for the analysis of metabolites by FIA-APPI-MS (Fig. 2). Besides the mixture methanol:water 1:1, other solvents usually employed in liquid chromatography mass spectrometry such as methanol and acetonitrile were tested, as well as isopropanol, a photoionizable solvent that may initiates ionization of analytes [27,42]. The solvent was delivered at a flow rate of 100 µl min^{-1}, and mixed with toluene at 10 µl min^{-1}. Compared with methanol:water, the use of pure methanol slightly increased sensitivity, according to previous works in which a reduction of the water content produced a general increase of sensitivity [47]. However, acetonitrile reduced the photoionization efficiency respect to methanol, probably due to more effective nebulization and vaporization processes provided by methanol [22] and the higher proton affinity (PA) of acetonitrile [24]. Finally, isopropanol produced comparable results to methanol, so it could be concluded that is not necessary to use photoionizable solvents if experimental conditions are well selected. The solvent flow is other important factor to be considered when the APPI source is employed, as it has been previously revised [17,48-49]. Using methanol as solvent and toluene as dopant, solvent flow was ranged between 50-500 µl min^{-1} maintaining the dopant flow at a 10%. The monitoring of the intensity for three representative standards depending on the flow is represented in Figure 3, which shows a different tendency when results from positive and negative ionization modes are compared. In positive mode, the maximum intensity was reached at low flow rates (about 50 µl min^{-1}), decreasing progressively when solvent flow increases. The loss of sensitivity at higher solvent flow rates can be attributed to different processes, such as the growth of ion-solvent clusters, because larger clusters may be unreactive and stimulate the loss of ions by recombination processes [17]. Furthermore, charge exchange is reduced due to the loss of dopant radical cations by reaction with the solvent molecules [48]. On the other hand, the highest ionization efficiency in negative mode is achieved with a solvent flow of 100 µl min^{-1} (Fig. 3b). Negative ionization is much less used in APPI, and the effect of solvent flow in photoionization has not been previously considered. However, negative ionization takes place through different mechanisms, such as electron capture, charge exchange, proton transfer, or substitution reactions [50-51], so it is not rare to find a different behavior according to the flow of solvent compared with positive mode. Finally, dopant flow was tested around 10-50% of the solvent flow previously selected. Thus, in positive ionization mode the dopant flow was ranged between 5-25 µl min^{-1}, but higher values were employed in negative mode, in the range of 10-50 µl min^{-1} (Fig. 4). Results show that ionization efficiency reaches a plateau at high dopant flows (around 30-40% of solvent flow) in both analysis modes. This finding is consistent with data presented by other authors for low flow applications, as is the case of the present study. Thereby, although under conventional LC conditions ionization efficiency quickly remains constant as more dopant is added, at about 5-10% of the solvent flow [17], it has been observed that this may result in a low rate of reagent ion production when solvent flow is reduced, so that the addition of more dopant may be advantageous [12]. Therefore, optimal dopant flows were set at 20 and 40 µl min^{-1}, for positive and negative modes of analysis, respectively.

APPLICATION OF FIA-APPI-MS TO A METABOLOMIC STUDY OF ALZHEIMER'S DISEASE

The suitability of the optimized APPI approach for the analysis of blood serum samples was confirmed in a metabolomic investigation of Alzheimer's disease. The potential of a two-steps methodology for serum extraction, complemented by the use of positive and negative ionization modes has been previously demonstrated for comprehensive metabolomic fingerprinting of serum samples by direct infusion electrospray mass spectrometry [40]. Similarly, analysis by FIA-APPI-MS of these serum extracts generated mass spectra with a high diversity of signals, as shown in Figure 5. It is noteworthy that for polar extracts the most prominent signals are observed at low m/z values, below 500 amu (Figs. 5a1 and 5a2), while in lipophilic extracts several clusters of peaks are obtained in the m/z range 500-1000 (Figs. 5b1 and 5b2), allowing a comprehensive assessment of serum metabolites. Then, results were submitted to partial least squares discriminant analysis (PLS-DA) in order to discriminate between AD patients and healthy controls. The figure 6 shows the scores plots of all the models, for APPI(+)-MS and APPI(-)-MS data from polar and lipophilic extracts, which provides a good classification of samples. The parameters R^2 and Q^2, listed in Table 3, were used to evaluate the performance of these models. As can be observed, satisfactory values for the quality parameters R^2 and Q^2 were obtained, with a variance explained around 100% and variance predicted above 80%, demonstrating the potential of flow injection analysis with APPI-MS for serum metabolomics. In addition, the validation plots from the permutation tests (not shown) demonstrated the validity of this discrimination given that Q^2 regression showed a negative intercept and R^2 values of permuted models were lower than the R^2 value of the original one, indicating that the models were not overfitted. Finally, metabolites responsible for discrimination were selected according to the Variable Importance in the Projection parameter (VIP>2). These discriminant metabolites are listed in Table 4, including several low molecular weight metabolites and different lipid classes. The reproducibility of the method was excellent, with coefficients of variation (CV) below 10% for all the metabolites identified (CV values were calculated from a sample analyzed in triplicate). Furthermore, it should be noted that all these compounds presented AUC values higher (or close) than 0.7, indicative of good diagnostic power.

METABOLIC ALTERATIONS IN SERUM FROM ALZHEIMER'S DISEASE

Metabolic fingerprints have shown important differences in serum from AD patients respect to healthy controls, considering up- or down-regulation of metabolites belonging to diverse classes, from lipids such as fatty acids or ceramides to amino acids and neurotransmitters. After the identification of altered metabolites, it is important to decipher disturbed cellular pathways in order to understand the biochemical processes underlying to disease. Thus, a tentative biochemical interpretation of results is presented in the next paragraphs.

Fatty acid amides and the endocannabinoid system

Primary fatty acid amides (PFAM) are bioactive lipids found in several tissues and biological fluids of mammals, including humans [46,52]. These compounds are important endogenous signaling molecules in the mammalian nervous system, binding to many receptors and demonstrating control over a variety of biological processes such as sleep regulation, modulation of monoaminergic systems, locomotion, inhibition of phospholipase A_2 (PLA_2) and epoxide hydrolase (EH), and many other processes [53-54]. Moreover, although these lipids do not interact with cannabinoid receptors, they are catabolically related to endocannabinoids and might regulate biological activities in mammals in a similar fashion [55]. Furthermore, PFAMs are also degraded by fatty acid amide hydrolase (FAAH) [56], the principal catabolic enzyme of the endocannabinoid system. In the context of Alzheimer's disease, there is growing evidence for the involvement of the endocannabinoid system in the development of neurodegeneration [57]. Thereby, the expression levels of cannabinoid receptors (CB) have been found altered in AD brains, including a decrease of CB1 receptors and up-regulation of CB2 receptors in the hippocampus [58]. In addition, a considerable over-expression of FAAH activity has been also associated with AD [59]. Therefore, the decrease of different fatty acid amides observed in this work (palmitoleamide, palmitamide, linolenamide, linoleamide, oleamide and stearamide) could be considered as an indirect marker of endocannabinoid dysregulation in Alzheimer's disease, as previously reported for serum oleamide [60].

Alterations in sphingolipid metabolism: increase of ceramides

The increase of serum ceramides (CER) and ceramide-1-phosphate (C1P) shown in Table 4 is consistent with impaired sphingolipid metabolism, whose involvement in AD has been extensively discussed [61]. The proper balance of sphingolipids is essential for neuronal function, given that sphingomyelins (SM) are critical components of lipid membrane rafts, and their catabolites are bioactive compounds and second messengers in cellular signaling and apoptosis. The sphingolipid pathway is altered at the gene expression level in AD, with up-regulated activities of enzymes controlling ceramide synthesis [62] and acid sphingomyelinase [63], suggesting a shift in metabolism

towards the accumulation of ceramides. Moreover, the contribution of ceramides to AD pathogenesis has been proposed on the basis of their activity as second messengers, being directly involved in apoptotic signaling, controlling secretion of APP and Aβ, or stimulating PLA_2 [64].Thereby, high levels of total ceramides have been previously associated with AD, mainly in post-mortem studies of brain tissue [62-63,65], but also in cerebrospinal fluid [66] and once in plasma [67], in agreement with our metabolomic findings.

Diacylglycerols and fatty acids: the role of phospholipases

The accumulation of diacylglycerols (DAGs) and free fatty acids (FFAs) in serum from AD patients (Table 4) has been previously associated with cellular membrane breakdown due to over-activation of phospholipases [60]. Abnormalities in membrane phospholipids in AD are principally related to altered expression of phospholipase A_2 (PLA_2) function [68], but there is also evidence for the role of phospholipases C and D, although they has been much less studied [69-70]. Furthermore, it is known the interconnections between the metabolism of phospholipids and sphingomyelins [71]. Ceramides and ceramide-1-phosphate induce overactivation of PLA_2, as stated in the previous section, while arachidonic acid (one of the most abundant fatty acids contained in neural phospholipids) released from the hydrolysis of these phospholipids by the action PLA_2, causes the overexpression of sphingomyelinase (SMase). Therefore, it could be concluded that membrane breakdown processes in Alzheimer's disease are finally reflected in multiple inter-related metabolites that are altered in serum samples, as schematized in Figure 7.

Involvement of low molecular weight metabolites

The involvement of low molecular weight metabolites in central pathways of metabolism makes them important targets in the study of pathological alterations underlying to diseases. In this study, the most important changes could be related to disturbances in neurotransmitter systems, given that decreased serum levels of serotonin, dopamine and taurine were found (Table 4). Serotonin and dopamine are two important monoaminergic neurotransmitters, whose altered synthesis have been previously associated with synaptic failure leading to neurodegeneration in Alzheimer's disease [72]. On the other hand, taurine presents several roles in neurotransmission, neuromodulation, osmoregulation, control of calcium influx and cell excitability, whose implication in neurodegeneration and AD has been previously demonstrated [73]. Hypometabolism is also documented in AD, with depleted neuronal energy production by decreased rate of carbohydrate catabolism [74] and oxidative deficiency characterized by mitochondrial dysfunction [75]. This energetic deficiency is in accordance with the altered levels of alanine, creatine and malate observed in metabolomic profiles (Table 4), given that these compounds participate in overall cellular bioenergetics. Picolinic acid is a tryptophan metabolite from the kynurenine pathway, which is up-regulated in AD by over-expression of indole-2,4-dioxygenase. Thereby, altered synthesis of related neuroactive compounds and the accumulation of different derivatives have been previously reported in AD [76], in agreement with the increased levels of picolinic acid observed in this study. Finally, the reduction of urea levels points to a perturbation of the urea cycle, responsible for controlling ammonia concentrations in the organism. The alteration of this pathway in AD has been previously demonstrated on the basis of altered levels of expression in different enzymes and the corresponding genes [77], which finally results in altered content of related metabolites as found in our metabolomic study.

CONCLUSIONS

A high throughput approach based on flow injection analysis and high resolution tandem mass spectrometry with atmospheric pressure photoionization source (FIA-APPI-QTOFMS) has been developed for metabolomic analysis of serum samples. Several experimental parameters affected considerably the ionization efficiency, such as dopant, carrier solvent or the flow rate of both. Toluene and methanol were selected as optimal dopant and flow injection solvent respectively, providing the highest sensitivity for the analysis of most of the metabolites. Considering the delivery speed of solvent and dopant, different behavior was observed for positive and negative analysis modes. In negative mode, the ionization was favored at higher solvent and dopant flows (100 µl min^{-1} and 40 µl min^{-1}), while in positive mode lower flows are recommended (50 µl min^{-1} and 20 µl min^{-1}). Then, the suitability of this APPI-MS technique was demonstrated in a metabolomic investigation of Alzheimer's disease. Eight low molecular weight metabolites and numerous lipids belonging to four different classes were detected as potential markers of disease, with good diagnostic power (AUC>0.7). Altered levels of primary fatty acid amides and major neurotransmitters such as serotonin or dopamine could be related to severe failures in neurotransmission. Moreover, important impairments associated with cellular membrane destabilization were also found, considering increased serum levels of ceramides, diacylglicerols and free fatty acids. Furthermore, other metabolic changes may be related to hypometabolism, hyperammonemia or up-regulation of the kynurenine pathway associated with this disease.

Although previous studies have suggested that blood biochemistry could reflect biochemical changes in the central nervous system, these findings should be confirmed in a next step in cerebrospinal fluid and/or post-mortem brain samples. Furthermore, as future plan, it would be interesting to extend this study to other types of dementia in order to assess the specificity of these potential markers against other neurodegenerative disorders.

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FIGURE CAPTIONS

Figure 1. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different dopants.
Figure 2. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different solvents.
Figure 3. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different solvent flows.
Figure 4. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different dopant flows.
Figure 5. Mass spectra from serum samples for APPI+ (1) and APPI- (2), with polar (a) and lipophilic (b) extracts.
Figure 6. Scores plots of the PLS-DA for APPI+ (1) and APPI– (2), with polar (a) and lipophilic (b) extracts. Black squares: Alzheimer's disease patients; red circles: healthy controls.
Figure 7. Membrane degradation and interconnections between metabolism of phospholipids and sphingolipids. ↑: compounds up-regulated, ↓: compounds down-regulated, dashed arrows: enzymatic stimulation

Table 1. Composition of the standard test mixture

Compound	**Concentration (µM)**
Creatinine (Crn)	60
Valine (Val)	230
Cysteine (Cys)	200
Creatine (Cr)	50
Aspartic acid (Asp)	20
Glutamine (Gln)	600
Glutamic acid (Glu)	50
Histidine (His)	90
Phenylalanine (Phe)	70
Arginine (Arg)	100
Glucose (Glc)	5000
Cholesterol (Chol)	5000
Di-oleoyl-phosphocholine (PC)	5
Triolein (TG)	30

Table 2. Acquisition time parameters according to the LC flow

LC flow (µl min^{-1})	**Delay time (sec)**	**Acquisition time (min)**
50	20	0.7
100	12	0.5
200	5	0.4
300	3	0.3
500	0	0.2

Table 3. Statistical parameters of the PLS-DA models

ion mode	**extract**	**R^2**	**Q^2**
APPI+	polar	0.998	0.849
	lipophilic	0.997	0.859
APPI-	polar	0.999	0.873
	lipophilic	1	0.883

Table 4. Discriminant metabolites identified in serum from Alzheimer's disease patients

compound	**MS/MS fragments**		**fold change**	**% CV**	**p value**	**AUC**
	APPI+	**APPI-**				
Fatty acid amides (PFAM)						
Palmitoleamide	254.22 $[M+H]^+$, 237.24, 219.23, 57.07	-	0.84	1.3	$8.6 \cdot 10^{-5}$	0.78

Palmitamide	256.24 $[M+H]^+$, 102.09, 88.08, 57.07	-	0.84	4.6	$4.5 \cdot 10^{-5}$	0.80
Linolenamide	278.22 $[M+H]^+$, 261.22, 243.21, 57.07	-	0.82	7.4	$2.0 \cdot 10^{-5}$	0.82
Linoleamide	280.25 $[M+H]^+$, 263.24, 245.22, 57.07	-	0.76	6.6	$5.5 \cdot 10^{-5}$	0.82
Oleamide	282.26 $[M+H]^+$, 265.26, 247.25, 57.07	-	0.72	8.8	$1.0 \cdot 10^{-6}$	0.90
Stearamide	284.27 $[M+H]^+$, 102.09, 88.08, 57.07	-	0.75	6.5	$3.6 \cdot 10^{-5}$	0.79
Ceramides (CER)						
CER(d18:1/16:1)	518.48 $[M+H-H_2O]^+$, 500.50, 282.27, 264.25	516.48 $[M-H-H_2O]^-$, 253.22	1.24	7.7	$1.7 \cdot 10^{-3}$	0.89
CER(d18:1/16:0)	520.50 $[M+H-H_2O]^+$, 502.50, 282.27, 264.25	518.49 $[M-H-H_2O]^-$, 255.22	1.28	3.5	$4.4 \cdot 10^{-4}$	0.90
CER(d18:1/18:1)	546.49 $[M+H-H_2O]^+$, 528.46, 282.27, 264.25	-	1.16	6.8	$2.0 \cdot 10^{-6}$	0.86
CER(d18:1/18:0)	-	546.46 $[M-H-H_2O]^-$, 283.26	1.56	2.3	$5.4 \cdot 10^{-3}$	0.70
CER(d18:1/18:0)-1P	628.57 $[M+H-H_2O]^+$, 610.56, 282.27, 264.25	626.56 $[M-H-H_2O]^-$, 283.26, 96.97	1.33	6.9	$7.5 \cdot 10^{-3}$	0.95
CER(d18:1/24:1)	630.60 $[M+H-H_2O]^+$, 612.56, 282.27, 264.25	-	1.39	6.2	$1.0 \cdot 10^{-6}$	0.92
Diacylglycerols (DAG)						
DAG(14:1/16:0)	539.42 $[M+H]^+$, 313.26, 283.25	-	1.21	7.7	$1.0 \cdot 10^{-6}$	0.85
DAG(16:0/18:3)	591.48 $[M+H]^+$, 335.26, 313.26, 261.20	-	1.38	6.8	$1.0 \cdot 10^{-6}$	0.85
DAG(16:0/18:1)	595.48 $[M+H]^+$, 339.29, 313.27, 265.22, 239.22	-	1.17	6.1	$3.0 \cdot 10^{-6}$	0.84
DAG(18:3/18:3)	613.50 $[M+H]^+$, 335.24	-	1.20	2.3	$3.0 \cdot 10^{-6}$	0.82
DAG(18:3/18:2)	615.48 $[M+H]^+$, 337.27, 335.24, 263.20, 261.20	-	1.22	7.9	$2.5 \cdot 10^{-4}$	0.75
DAG(16:0/20:4)	617.50 $[M+H]^+$, 361.33, 313.27, 287.21	-	1.25	5.3	$1.5 \cdot 10^{-4}$	0.76
DAG(18:2/18:1)	619.51 $[M+H]^+$, 339.29, 337.24	-	1.26	3.2	$1.2 \cdot 10^{-4}$	0.75
Free fatty acids (FFA)						
Palmitoleic acid	-	253.22 $[M-H]^-$	1.49	5.1	$7.2 \cdot 10^{-4}$	0.81
Palmitic acid	-	255.22 $[M-H]^-$	1.36	10.6	$1.3 \cdot 10^{-3}$	0.78
Oleic acid	-	281.24 $[M-H]^-$	1.44	6.7	$5.4 \cdot 10^{-4}$	0.80
Low molecular weight metabolites						
Urea	61.04 $[M+H]^+$, 44.02	-	0.76	4.1	$9.9 \cdot 10^{-3}$	0.68
Alanine	90.06 $[M+H]^+$, 44.04	-	1.43	2.9	$1.0 \cdot 10^{-6}$	0.92
Taurine	-	124.00 $[M-H]^-$, 79.97	0.88	11.6	$2.7 \cdot 10^{-3}$	0.73
Picolinic acid	124.05 $[M+H]^+$, 106.02, 78.03	-	1.15	4.1	$4.1 \cdot 10^{-5}$	0.80
Creatine	-	130.06 $[M-H]^-$, 88.05	0.87	0.9	$4.9 \cdot 10^{-3}$	0.69
Malic acid	-	133.02 $[M-H]^-$, 115.03, 71.02	0.88	3.6	$9.7 \cdot 10^{-4}$	0.84
Dopamine	136.07 $[M+H-H_2O]^+$, 119.05, 91.04	-	0.77	3.3	$1.0 \cdot 10^{-6}$	0.93
Serotonin	177.09 $[M-H]^-$, 160.07, 115.05	-	0.90	3.3	$1.2 \cdot 10^{-2}$	0.89

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APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE

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The use of atmospheric pressure photoionization is not widespread in metabolomics, despite its considerable potential for the simultaneous analysis of compounds with diverse polarities. This work considers the development of a novel analytical approach based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for rapid metabolic screening of serum samples. Several experimental parameters were optimized, such as type of dopant, flow injection solvent, and their flows, given that a careful selection of these variables is mandatory for a comprehensive analysis of metabolites. Toluene and methanol were the most suitable dopant and flow injection solvent, respectively. Moreover, analysis in negative mode required higher solvent and dopant flows (100 µl min^{-1} and 40 µl min^{-1}, respectively) compared to positive mode (50 µl min^{-1} and 20 µl min^{-1}). Then, the optimized approach was used to elucidate metabolic alterations associated with Alzheimer's disease. Thereby, results confirm the increase of diacylglycerols, ceramides, ceramide-1-phosphate and free fatty acids, indicating membrane destabilization processes, and reduction of fatty acid amides and several neurotransmitters related to impairments in neuronal transmission, among others. Therefore, it could be concluded that this metabolomic tool presents a great potential for analysis of biological samples, considering its high-throughput screening capability, fast analysis and comprehensive metabolite coverage.

Keywords
Alzheimer's disease, metabolomics, atmospheric pressure photoionization, flow injection.

Novelty statement
This work describes for the first time the use of a metabolomic platform based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for the investigation of metabolic alterations associated with Alzheimer's disease.

INTRODUCTION

The main challenge in metabolomics is to obtain comprehensive and unbiased metabolic fingerprints of samples due to the huge heterogeneity and dynamism of metabolome [1]. In this context, mass spectrometry represents a very interesting analytical platform, since complexity of metabolome may be overcome through the use of complementary atmospheric pressure ionization methods [2]. Electrospray (ESI) is the most common ionization source employed in metabolomic studies because it is able to ionize compounds in a wide range of masses and polarities, and it may be coupled to liquid chromatography [3-4], capillary electrophoresis [5-6] or used by direct infusion mass spectrometry [7]. A second alternative is atmospheric pressure chemical ionization (APCI), more suitable for less polar compounds [8-9]. Finally, atmospheric pressure photoionization (APPI) complements ESI and APCI for the analysis of little polar or non-polar compounds, but it has been considerably less used in metabolomics. Atmospheric pressure photoionization is the most recent soft ionization technique for mass spectrometry, introduced simultaneously by Bruins and Syage in 2000 [10-11], extending the range of ionizable compounds to less polar ones, which are not readily ionized by ESI and APCI. Nevertheless, APPI is capable of ionizing both polar and non-polar compounds through proton transfer and charge exchange reactions respectively, so it could be considered a universal ionization source [12]. The APPI interface uses a photoionization lamp and a dopant flow to form dopant radical ions, which can directly ionize non-polar analytes through charge exchange reactions. On the other hand, for polar compounds dopant photoions can produce intermediate reactive species by reactions with solvent or oxygen molecules, in positive and negative ionization modes respectively, followed by a proton transfer reaction to the analyte [12]. Thereby, for the simultaneous analysis of both polar and non-polar compounds, the two reaction pathways must be accessible, which requires a careful selection of solvent, dopant, and their flows. Firstly, the carrier solvent employed can lead to a preferential mechanism, being favored charge exchange for low proton affinity solvents, whereas the addition of methanol or acetonitrile initiates proton transfer [13]. In the case of dopant, the most common reagent is toluene, but it may be not suitable for the ionization of non polar compounds in high proton affinity solvents since tends to transfer its proton to the solvent [14]. Alternatively, other dopants less reactive with the solvent have been proposed, such as anisole [15] or substituted benzenes [16]. Finally, the flow-rate of both solvent and dopant also play important roles in the photoionization process. In the case of solvent, ionization efficiency decreases when the flow increases, due to the formation of large unreactive protonated-solvent cluster ions, while signal increases with the dopant flow, until reach 5-10% of solvent flow [17]. Moreover, it has been reported that toluene can provide high ionization efficiency simultaneously to both polar and non-polar compounds delivered in reversed-phase solvents, simply limiting the solvent flow rate in order to avoid that reactions between toluene photoions and solvent were driven to completion [18].

In this way, APPI might present a considerable potential in metabolomics, not only because of its universality in terms of ionization capability, but also because is less susceptible to matrix effects and presents a linear dynamic range generally higher than that for ESI [19-20]. In addition, it requires less heat for desolvation than APCI allowing the analysis of thermally labile compounds [21]. APPI has been used for the analysis of many classes of compounds, including pharmaceutical drugs and metabolites [22], steroids [21], aldehydes and ketones [23] or pesticides [24]. However, only a few non targeted approaches include APPI as an alternative in metabolomics. Thereby, metabolomics based on liquid chromatography mass spectrometry and the integration of multiple ionization modes (ESI, APCI and APPI) has been previously proposed for the study of urine [25] and plasma samples [26], or to perform a more comprehensive analysis of lipidome [27-28]. Although APPI is normally coupled to liquid chromatography [29] or capillary electrophoresis [30], several reports confirm the ability of flow injection analysis for the determination of fullerenes and perfluorinated compounds [31], characterization of wine [32], olive oil [33] or Iberian ham [34], or the study of drugs [35] and petroleum [36]. This high-throughput approach exhibits several advantages in metabolomics such as fast and reproducible analysis, comprehensive metabolite coverage and simple data pre-processing [7], but it also presents important drawbacks associated with the lack of resolution for the differentiation of isobars and difficulty of quantification due to ion suppression. In order to overcome problems associated with isobaric interferences, the use of high resolution systems has become the main workhorse for accurate MS-fingerprinting, including time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR), and especially the hybrid system Q-TOF, which allows more accurate mass measurement than single TOF instruments and structural elucidation by MS/MS experiments [37]. On the other hand, although ion suppression is a potential problem in any MS-based metabolomic platform, there is no evidence that it presents a more detrimental effect on flow infusion fingerprinting than in hyphenated approaches [7]. In fact, the large amount of different compounds in biological samples helps to regulate matrix effects, so that ion suppression becomes a constant factor imposed uniformly in all samples derived from similar types of tissues [38]. Thereby, MS-fingerprinting has proved to be an excellent tool for high-throughput metabolomic characterization of complex samples, usually using electrospray

ionization, as recently reviewed by Draper et al. [7]. However, to date, the use of FIA-APPI-MS has not been considered in metabolomic analysis.

This work explores for the first time the potential of flow injection analysis and high resolution tandem mass spectrometry with atmospheric pressure photoionization source (FIA-APPI-QTOFMS) in metabolomics. Several critical parameters were optimized for the simultaneous analysis of both polar and non-polar compounds in positive and negative ionization modes, such as type of dopant, flow injection solvent and their working flows. For this purpose, a test mixture of representative metabolites from human serum was used. Then, the optimized approach was applied to blood serum samples in order to investigate metabolic abnormalities associated with Alzheimer's disease (AD). This neurodegenerative disorder is poorly understood, and its etiology is still unknown, although it is likely to be a conglomeration of different pathological entities. There is currently no cure for Alzheimer's disease, but early diagnosis could help monitor disease progression and target therapies earlier in the course of the disease, so identification of reliable biomarkers is becoming increasingly important. Multivariate statistics demonstrated the ability of this high-throughput metabolomic tool for discriminating between AD patients and healthy controls, and allowed the identification of different metabolic failures underlying to pathological features of this health disorder.

MATERIAL AND METHODS

CHEMICALS AND SAMPLES

The solvents (HPLC-grade) methanol, ethanol, chloroform, dichlorometane, acetonitrile and isopropanol were purchased from Fisher Scientific (Leicestershire, UK), while dopants toluene, anisole and chlorobenzene were supplied by Aldrich (Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Standards of L-glutamine, L-valine, L-cysteine, L-aspartic acid, L-arginine, L-histidine, L-glutamic acid, L-phenylalanine, D-glucose, creatine, creatinine, cholesterol, di-oleoyl-phosphocholine and triolein were from Sigma Aldrich. Blood samples were collected in the morning by puncture of the antecubital vein and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. The samples were immediately cooled and protected from light for 30 minutes, and after centrifugation (3500 rpm for 10 minutes) serum was aliquoted and frozen at -80°C until analysis. Alzheimer's disease patients (AD, N=30, age 80.3±5.0, male/female 12/18) were newly diagnosed of sporadic Alzheimer's disease by the Neurologic Service of Hospital Juan Ramón Jiménez (Huelva, Spain), according to the criteria of the NINCDS-ADRDA [39], and only subjects that had not yet received any type of medication were included in the study. Healthy controls (HC, N=30, age 73.5±5.9, male/female 10/20), who had not more than two reported cases of Alzheimer's disease in their families, were studied by neurologists to confirm the absence of neurological disorders. Demographic characteristics of groups considered in the study are listed in the Supporting Information, including age, gender, co-morbidities, medication and family history of AD. It is noteworthy that most subjects suffered other co-morbidities and were under different medical treatments, but there were not significant differences among the groups considered. The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of University of Huelva.

PREPARATION OF SAMPLES

Optimization of experimental conditions was performed with a solution containing standards of representative metabolites from blood serum in methanol:water (1:1, v/v), including different low molecular weight compounds from different chemical classes and lipids, ranging diverse acid-base properties and polarities. The composition of this mixture was selected according to their usual concentrations in human serum described in the Human Metabolome Database (HMDB), as shown in Table 1. This metabolomic approach was further tested in real serum, for which samples were treated in a two-stage sequential procedure described elsewhere for comprehensive serum metabolomics [40]. For the extraction of metabolites, 100 µL of serum are mixed with 400 µL of methanol/ethanol 1:1 and stirred for 5 min, followed by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant is transferred to another tube, and the precipitate is kept for further treatment. Then, supernatant is dried under nitrogen stream and the resulting residue reconstituted with 80 µL of methanol and 20 µL of water (polar extract). On the other hand, the precipitate isolated in the first step is extracted with 400 µL of chloroform:methanol 1:1 by stirring during 5 min, followed by centrifugation at 10000 rpm for 10 minutes at 4 °C. Finally, the resulting supernatant is taken to dryness under nitrogen stream and reconstituted with 100 microliters of 60:40 dichloromethane:methanol (lipophilic extract).

INSTRUMENTATION

Mass spectrometry experiments were performed on a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure photoionization (APPI) source. Samples were introduced by flow injection using an Accela LC system (Thermo Fisher Scientific) equipped with autosampler and quaternary pump. In

addition, a model KDS 100 syringe pump from KD scientific (New Hope, PA, USA) was employed to deliver the dopant for photospray ionization. For accurate mass measurement, the TOF analyzer is calibrated before analyzing each batch of samples using renin as standard. Data were obtained both in positive and negative ion modes, injecting 10 µl of sample, and acquiring full scan spectra in the m/z range 50-1100 with 1.005 seconds of scan time. Acquisition time was selected according to the LC flow employed to consider the dispersion of the plug of sample into the solvent stream, and employing some delay time between injection and analysis to allow enough time for the sample to reach the mass spectrometer (settings shown in Table 2). The ion spray voltage (IS) was set at 1500 V and -2300 V in positive and negative modes respectively, with declustering potential (DP) of ±50 V and focusing potential (FP) of ±250 V. The source temperature was maintained at 400ºC, and the gas flows (high-purity nitrogen) were fixed at 1.13 L min^{-1} for curtain gas, 1.50 L min^{-1} for nebulizer gas, 3.0 L min^{-1} for heater gas, and 1 L min^{-1} for lamp gas. To acquire MS/MS spectra, nitrogen was used as collision gas and the collision energy was ranged between 10-60 V. The optimization of FIA conditions was performed in four steps: type of dopant, type of carrier solvent, solvent flow and, finally, dopant flow. For the analysis of serum samples, the optimal dopant was toluene delivered at 20/40 µl min^{-1}, in positive and negative ion mode respectively. Furthermore, methanol was used as flow injection solvent at 50/100 µl min^{-1}, in both ionization modes.

STATISTICAL ANALYSIS

Metabolomic data were submitted to peak detection by Markerview™ software (Applied Biosystems) in order to filter the mass spectrometry results, and to carry out the reduction into a two-dimensional data matrix of spectral peaks and their intensities. For this, the peak search was done with a mass tolerance of 0.1Da, and a minimum response of 10 counts was considered for filtering. Finally, data were normalized according to the total area sum. Then, data was processed in SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden) in order to find differences between the groups of study (AD patients and healthy controls). Partial least squares discriminant analysis (PLS-DA) was performed to build predictive models that provide class separation and allow the further study of discriminant metabolites. Quality of the models was assessed by the R^2 and Q^2 values, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). In addition, these models were validated using permutation tests (Y-scrambling) of the Y-predicted values. In Y-scrambling, class labels are randomly permuted for refitting a new model with the same number of components as the original one, and then these new models are compared with the original models to test the possibility that the original model arose by chance. Thus, an overfitted model will have similar R^2 and Q^2 to that of the randomly permuted data, while well fitted and meaningful models will have R^2 and Q^2 values higher than that of the permuted data. Finally, discriminant compounds were selected according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 2.0, indicative of significant differences among groups. Furthermore, these potential biomarkers were subjected to receiver operating characteristic (ROC) analysis to assess their diagnostic ability. The ROC curve analysis was performed using the GraphPad Prism software (version 6.04, Intuitive Software for Science, San Diego, CA), and the area under the curve (AUC) was used as a metric of sensitivity and specificity of these biomarkers. Thereby, a marker is excellent when AUC ranges from 0.9 to 1, good if AUC is 0.8 to 0.9, moderate if AUC is 0.8 to 0.7 and poor below 0.7 [41].

IDENTIFICATION OF METABOLITES

Discriminant metabolites were identified matching the experimental accurate mass and tandem mass spectra (MS/MS) with those available in metabolomic databases (HMDB, METLIN, LIPIDMAPS), using a mass accuracy of 50 ppm. Then, different classes of lipids were confirmed based on characteristic fragmentation patterns previously described. It is noteworthy that signals from APPI mass spectra can be mainly attributed to protonated or deprotonated adducts (in positive and negative modes, respectively), but the possibility to produce dehydrated molecular ions has been also previously demonstrated [42-44]. Ceramides presented characteristic product ions in positive ionization mode at m/z 264 and 282 due to the fragmentation in the sphingosine moiety [45]. On the other hand, fragmentation of glycerolipids (diacylglycerols) occurs through the release of fatty acids generating different types of ions (named A, B, C and D), which show characteristic m/z values according to the fatty acid attached to the glycerol backbone [34]. Finally, fatty acid amides were confirmed with characteristic fragments described in the literature [46].

RESULTS AND DISCUSSION

SELECTION OF EXPERIMENTAL CONDITIONS

The effect of experimental conditions on the photoionization efficiency was evaluated by flow injection analysis of a mixture solution of representative metabolites (Table 1), acquiring in both positive and negative ion modes. Firstly, for the selection of dopant the other parameters were adjusted according to previous studies for the simultaneous determination of both polar and non-polar compounds [18], employing a mixture of methanol:water (1:1, v/v) as solvent at a flow rate of 100 µl min^{-1}. Dopant flow was fixed at a 10% of solvent flow (10 µl min^{-1}), since a plateau in ionization efficiency is reached in this source as more dopant is added [17]. The most common dopants described in literature were tested in this study (i.e. toluene, chlorobenzene and anisole), and the ionization efficiencies were compared in function of peak intensities (Fig. 1). In positive ionization mode (Fig. 1a) the best sensitivity was obtained with toluene for most of the standards, followed by chlorobenzene (it should be noted that the great increase in intensity observed for histidine with chlorobenzene is attributable to the presence of an impurity in this solvent with the same mass, which was checked using a blank test). However, for lipid compounds the peak intensity is slightly increased with anisole and chlorobenzene, although none of these dopants allowed the ionization of di-oleoyl-phosphocholine. On the other hand, intensities of low molecular weight metabolites were very similar in negative mode using the three dopants, although generally lower than that in positive mode, while lipids were not detected. The enhanced ionization observed with toluene and chlorobenzene compared to anisole could be due to their higher ionization energy (IE) values (8.83, 9.07, and 8.20 eV, respectively), given that for an effective photoionization the IE of the dopant must be higher than that of the analytes. Moreover, the better sensitivity of toluene compared to chlorobenzene for polar metabolites in positive mode, despite its lower IE, may be due to the impossibility of chlorobenzene to perform photoinozation by proton transfer [16]. Conversely, this low reactivity of chlorobenzene and anisole with the solvent is also responsible for the increased signal of non-polar analytes such as cholesterol and triolein using these dopants, although good results were also obtained with toluene. Therefore, toluene was selected as dopant for further experiences, since it provides acceptable results with most of metabolites with the highest mean sensitivity. In a second step, different solvents were evaluated for the analysis of metabolites by FIA-APPI-MS (Fig. 2). Besides the mixture methanol:water 1:1, other solvents usually employed in liquid chromatography mass spectrometry such as methanol and acetonitrile were tested, as well as isopropanol, a photoionizable solvent that may initiates ionization of analytes [27,42]. The solvent was delivered at a flow rate of 100 µl min^{-1}, and mixed with toluene at 10 µl min^{-1}. Compared with methanol:water, the use of pure methanol slightly increased sensitivity, according to previous works in which a reduction of the water content produced a general increase of sensitivity [47]. However, acetonitrile reduced the photoionization efficiency respect to methanol, probably due to more effective nebulization and vaporization processes provided by methanol [22] and the higher proton affinity (PA) of acetonitrile [24]. Finally, isopropanol produced comparable results to methanol, so it could be concluded that is not necessary to use photoionizable solvents if experimental conditions are well selected. The solvent flow is other important factor to be considered when the APPI source is employed, as it has been previously revised [17,48-49]. Using methanol as solvent and toluene as dopant, solvent flow was ranged between 50-500 µl min^{-1} maintaining the dopant flow at a 10%. The monitoring of the intensity for three representative standards depending on the flow is represented in Figure 3, which shows a different tendency when results from positive and negative ionization modes are compared. In positive mode, the maximum intensity was reached at low flow rates (about 50 µl min^{-1}), decreasing progressively when solvent flow increases. The loss of sensitivity at higher solvent flow rates can be attributed to different processes, such as the growth of ion-solvent clusters, because larger clusters may be unreactive and stimulate the loss of ions by recombination processes [17]. Furthermore, charge exchange is reduced due to the loss of dopant radical cations by reaction with the solvent molecules [48]. On the other hand, the highest ionization efficiency in negative mode is achieved with a solvent flow of 100 µl min^{-1} (Fig. 3b). Negative ionization is much less used in APPI, and the effect of solvent flow in photoionization has not been previously considered. However, negative ionization takes place through different mechanisms, such as electron capture, charge exchange, proton transfer, or substitution reactions [50-51], so it is not rare to find a different behavior according to the flow of solvent compared with positive mode. Finally, dopant flow was tested around 10-50% of the solvent flow previously selected. Thus, in positive ionization mode the dopant flow was ranged between 5-25 µl min^{-1}, but higher values were employed in negative mode, in the range of 10-50 µl min^{-1} (Fig. 4). Results show that ionization efficiency reaches a plateau at high dopant flows (around 30-40% of solvent flow) in both analysis modes. This finding is consistent with data presented by other authors for low flow applications, as is the case of the present study. Thereby, although under conventional LC conditions ionization efficiency quickly remains constant as more dopant is added, at about 5-10% of the solvent flow [17], it has been observed that this may result in a low rate of reagent ion production when solvent flow is reduced, so that the addition of more dopant may be advantageous [12]. Therefore, optimal dopant flows were set at 20 and 40 µl min^{-1} for positive and negative modes of analysis, respectively.

APPLICATION OF FIA-APPI-MS TO A METABOLOMIC STUDY OF ALZHEIMER'S DISEASE

The suitability of the optimized APPI approach for the analysis of blood serum samples was confirmed in a metabolomic investigation of Alzheimer's disease. The potential of a two-steps methodology for serum extraction, complemented by the use of positive and negative ionization modes has been previously demonstrated for comprehensive metabolomic fingerprinting of serum samples by direct infusion electrospray mass spectrometry [40]. Similarly, analysis by FIA-APPI-MS of these serum extracts generated mass spectra with a high diversity of signals, as shown in Figure 5. It is noteworthy that for polar extracts the most prominent signals are observed at low m/z values, below 500 amu (Figs. 5a1 and 5a2), while in lipophilic extracts several clusters of peaks are obtained in the m/z range 500-1000 (Figs. 5b1 and 5b2), allowing a comprehensive assessment of serum metabolites. Then, results were submitted to partial least squares discriminant analysis (PLS-DA) in order to discriminate between AD patients and healthy controls. The figure 6 shows the scores plots of all the models, for APPI(+)-MS and APPI(-)-MS data from polar and lipophilic extracts, which provides a good classification of samples. The parameters R^2 and Q^2, listed in Table 3, were used to evaluate the performance of these models. As can be observed, satisfactory values for the quality parameters R^2 and Q^2 were obtained, with a variance explained around 100% and variance predicted above 80%, demonstrating the potential of flow injection analysis with APPI-MS for serum metabolomics. In addition, the validation plots from the permutation tests (not shown) demonstrated the validity of this discrimination given that Q^2 regression showed a negative intercept and R^2 values of permuted models were lower than the R^2 value of the original one, indicating that the models were not overfitted. Finally, metabolites responsible for discrimination were selected according to the Variable Importance in the Projection parameter (VIP>2). These discriminant metabolites are listed in Table 4, including several low molecular weight metabolites and different lipid classes. The reproducibility of the method was excellent, with coefficients of variation (CV) below 10% for all the metabolites identified (CV values were calculated from a sample analyzed in triplicate). Furthermore, it should be noted that all these compounds presented AUC values higher (or close) than 0.7, indicative of good diagnostic power.

METABOLIC ALTERATIONS IN SERUM FROM ALZHEIMER'S DISEASE

Metabolic fingerprints have shown important differences in serum from AD patients respect to healthy controls, considering up- or down-regulation of metabolites belonging to diverse classes, from lipids such as fatty acids or ceramides to amino acids and neurotransmitters. After the identification of altered metabolites, it is important to decipher disturbed cellular pathways in order to understand the biochemical processes underlying to disease. Thus, a tentative biochemical interpretation of results is presented in the next paragraphs.

Fatty acid amides and the endocannabinoid system

Primary fatty acid amides (PFAM) are bioactive lipids found in several tissues and biological fluids of mammals, including humans [46,52]. These compounds are important endogenous signaling molecules in the mammalian nervous system, binding to many receptors and demonstrating control over a variety of biological processes such as sleep regulation, modulation of monoaminergic systems, locomotion, inhibition of phospholipase A_2 (PLA_2) and epoxide hydrolase (EH), and many other processes [53-54]. Moreover, although these lipids do not interact with cannabinoid receptors, they are catabolically related to endocannabinoids and might regulate biological activities in mammals in a similar fashion [55]. Furthermore, PFAMs are also degraded by fatty acid amide hydrolase (FAAH) [56], the principal catabolic enzyme of the endocannabinoid system. In the context of Alzheimer's disease, there is growing evidence for the involvement of the endocannabinoid system in the development of neurodegeneration [57]. Thereby, the expression levels of cannabinoid receptors (CB) have been found altered in AD brains, including a decrease of CB1 receptors and up-regulation of CB2 receptors in the hippocampus [58]. In addition, a considerable over-expression of FAAH activity has been also associated with AD [59]. Therefore, the decrease of different fatty acid amides observed in this work (palmitoleamide, palmitamide, linolenamide, linoleamide, oleamide and stearamide) could be considered as an indirect marker of endocannabinoid dysregulation in Alzheimer's disease, as previously reported for serum oleamide [60].

Alterations in sphingolipid metabolism: increase of ceramides

The increase of serum ceramides (CER) and ceramide-1-phosphate (C1P) shown in Table 4 is consistent with impaired sphingolipid metabolism, whose involvement in AD has been extensively discussed [61]. The proper balance of sphingolipids is essential for neuronal function, given that sphingomyelins (SM) are critical components of lipid membrane rafts, and their catabolites are bioactive compounds and second messengers in cellular signaling and apoptosis. The sphingolipid pathway is altered at the gene expression level in AD, with up-regulated activities of enzymes controlling ceramide synthesis [62] and acid sphingomyelinase [63], suggesting a shift in metabolism

towards the accumulation of ceramides. Moreover, the contribution of ceramides to AD pathogenesis has been proposed on the basis of their activity as second messengers, being directly involved in apoptotic signaling, controlling secretion of APP and Aβ, or stimulating PLA_2 [64].Thereby, high levels of total ceramides have been previously associated with AD, mainly in post-mortem studies of brain tissue [62-63,65], but also in cerebrospinal fluid [66] and once in plasma [67], in agreement with our metabolomic findings.

Diacylglycerols and fatty acids: the role of phospholipases

The accumulation of diacylglycerols (DAGs) and free fatty acids (FFAs) in serum from AD patients (Table 4) has been previously associated with cellular membrane breakdown due to over-activation of phospholipases [60]. Abnormalities in membrane phospholipids in AD are principally related to altered expression of phospholipase A_2 (PLA_2) function [68], but there is also evidence for the role of phospholipases C and D, although they has been much less studied [69-70]. Furthermore, it is known the interconnections between the metabolism of phospholipids and sphingomyelins [71]. Ceramides and ceramide-1-phosphate induce overactivation of PLA_2, as stated in the previous section, while arachidonic acid (one of the most abundant fatty acids contained in neural phospholipids) released from the hydrolysis of these phospholipids by the action PLA_2, causes the overexpression of sphingomyelinase (SMase). Therefore, it could be concluded that membrane breakdown processes in Alzheimer's disease are finally reflected in multiple inter-related metabolites that are altered in serum samples, as schematized in Figure 7.

Involvement of low molecular weight metabolites

The involvement of low molecular weight metabolites in central pathways of metabolism makes them important targets in the study of pathological alterations underlying to diseases. In this study, the most important changes could be related to disturbances in neurotransmitter systems, given that decreased serum levels of serotonin, dopamine and taurine were found (Table 4). Serotonin and dopamine are two important monoaminergic neurotransmitters, whose altered synthesis have been previously associated with synaptic failure leading to neurodegeneration in Alzheimer's disease [72]. On the other hand, taurine presents several roles in neurotransmission, neuromodulation, osmoregulation, control of calcium influx and cell excitability, whose implication in neurodegeneration and AD has been previously demonstrated [73]. Hypometabolism is also documented in AD, with depleted neuronal energy production by decreased rate of carbohydrate catabolism [74] and oxidative deficiency characterized by mitochondrial dysfunction [75]. This energetic deficiency is in accordance with the altered levels of alanine, creatine and malate observed in metabolomic profiles (Table 4), given that these compounds participate in overall cellular bioenergetics. Picolinic acid is a tryptophan metabolite from the kynurenine pathway, which is up-regulated in AD by over-expression of indole-2,4-dioxygenase. Thereby, altered synthesis of related neuroactive compounds and the accumulation of different derivatives have been previously reported in AD [76], in agreement with the increased levels of picolinic acid observed in this study. Finally, the reduction of urea levels points to a perturbation of the urea cycle, responsible for controlling ammonia concentrations in the organism. The alteration of this pathway in AD has been previously demonstrated on the basis of altered levels of expression in different enzymes and the corresponding genes [77], which finally results in altered content of related metabolites as found in our metabolomic study.

CONCLUSIONS

A high throughput approach based on flow injection analysis and high resolution tandem mass spectrometry with atmospheric pressure photoionization source (FIA-APPI-QTOFMS) has been developed for metabolomic analysis of serum samples. Several experimental parameters affected considerably the ionization efficiency, such as dopant, carrier solvent or the flow rate of both. Toluene and methanol were selected as optimal dopant and flow injection solvent respectively, providing the highest sensitivity for the analysis of most of the metabolites. Considering the delivery speed of solvent and dopant, different behavior was observed for positive and negative analysis modes. In negative mode, the ionization was favored at higher solvent and dopant flows (100 µl min^{-1} and 40 µl min^{-1}), while in positive mode lower flows are recommended (50 µl min^{-1} and 20 µl min^{-1}). Then, the suitability of this APPI-MS technique was demonstrated in a metabolomic investigation of Alzheimer's disease. Eight low molecular weight metabolites and numerous lipids belonging to four different classes were detected as potential markers of disease, with good diagnostic power (AUC>0.7). Altered levels of primary fatty acid amides and major neurotransmitters such as serotonin or dopamine could be related to severe failures in neurotransmission. Moreover, important impairments associated with cellular membrane destabilization were also found, considering increased serum levels of ceramides, diacylglicerols and free fatty acids. Furthermore, other metabolic changes may be related to hypometabolism, hyperammonemia or up-regulation of the kynurenine pathway associated with this disease.

Although previous studies have suggested that blood biochemistry could reflect biochemical changes in the central nervous system, these findings should be confirmed in a next step in cerebrospinal fluid and/or post-mortem brain samples. Furthermore, as future plan, it would be interesting to extend this study to other types of dementia in order to assess the specificity of these potential markers against other neurodegenerative disorders.

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FIGURE CAPTIONS

Figure 1. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different dopants.
Figure 2. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different solvents.
Figure 3. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different solvent flows.
Figure 4. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different dopant flows.
Figure 5. Mass spectra from serum samples for APPI+ (1) and APPI- (2), with polar (a) and lipophilic (b) extracts.
Figure 6. Scores plots of the PLS-DA for APPI+ (1) and APPI– (2), with polar (a) and lipophilic (b) extracts. Black squares: Alzheimer's disease patients; red circles: healthy controls.
Figure 7. Membrane degradation and interconnections between metabolism of phospholipids and sphingolipids. ↑: compounds up-regulated, ↓: compounds down-regulated, dashed arrows: enzymatic stimulation

Table 1. Composition of the standard test mixture

Compound	**Concentration (µM)**
Creatinine (Crn)	60
Valine (Val)	230
Cysteine (Cys)	200
Creatine (Cr)	50
Aspartic acid (Asp)	20
Glutamine (Gln)	600
Glutamic acid (Glu)	50
Histidine (His)	90
Phenylalanine (Phe)	70
Arginine (Arg)	100
Glucose (Glc)	5000
Cholesterol (Chol)	5000
Di-oleoyl-phosphocholine (PC)	5
Triolein (TG)	30

Table 2. Acquisition time parameters according to the LC flow

LC flow (µl min^{-1})	**Delay time (sec)**	**Acquisition time (min)**
50	20	0.7
100	12	0.5
200	5	0.4
300	3	0.3
500	0	0.2

Table 3. Statistical parameters of the PLS-DA models

ion mode	**extract**	**R^2**	**Q^2**
APPI+	polar	0.998	0.849
	lipophilic	0.997	0.859
APPI-	polar	0.999	0.873
	lipophilic	1	0.883

Table 4. Discriminant metabolites identified in serum from Alzheimer's disease patients

compound	**MS/MS fragments**		**fold change**	**% CV**	**p value**	**AUC**
	APPI+	**APPI-**				
Fatty acid amides (PFAM)						
Palmitoleamide	254.22 $[M+H]^+$, 237.24, 219.23, 57.07	-	0.84	1.3	$8.6 \cdot 10^{-5}$	0.78

Palmitamide	256.24 $[M+H]^+$, 102.09, 88.08, 57.07	-	0.84	4.6	$4.5 \cdot 10^{-5}$	0.80
Linolenamide	278.22 $[M+H]^+$, 261.22, 243.21, 57.07	-	0.82	7.4	$2.0 \cdot 10^{-5}$	0.82
Linoleamide	280.25 $[M+H]^+$, 263.24, 245.22, 57.07	-	0.76	6.6	$5.5 \cdot 10^{-5}$	0.82
Oleamide	282.26 $[M+H]^+$, 265.26, 247.25, 57.07	-	0.72	8.8	$1.0 \cdot 10^{-6}$	0.90
Stearamide	284.27 $[M+H]^+$, 102.09, 88.08, 57.07	-	0.75	6.5	$3.6 \cdot 10^{-5}$	0.79
Ceramides (CER)						
CER(d18:1/16:1)	518.48 $[M+H-H_2O]^+$, 500.50, 282.27, 264.25	516.48 $[M-H-H_2O]^-$, 253.22	1.24	7.7	$1.7 \cdot 10^{-3}$	0.89
CER(d18:1/16:0)	520.50 $[M+H-H_2O]^+$, 502.50, 282.27, 264.25	518.49 $[M-H-H_2O]^-$, 255.22	1.28	3.5	$4.4 \cdot 10^{-4}$	0.90
CER(d18:1/18:1)	546.49 $[M+H-H_2O]^+$, 528.46, 282.27, 264.25	-	1.16	6.8	$2.0 \cdot 10^{-6}$	0.86
CER(d18:1/18:0)	-	546.46 $[M-H-H_2O]^-$, 283.26	1.56	2.3	$5.4 \cdot 10^{-3}$	0.70
CER(d18:1/18:0)-1P	628.57 $[M+H-H_2O]^+$, 610.56, 282.27, 264.25	626.56 $[M-H-H_2O]^-$, 283.26, 96.97	1.33	6.9	$7.5 \cdot 10^{-3}$	0.95
CER(d18:1/24:1)	630.60 $[M+H-H_2O]^+$, 612.56, 282.27, 264.25	-	1.39	6.2	$1.0 \cdot 10^{-6}$	0.92
Diacylglycerols (DAG)						
DAG(14:1/16:0)	539.42 $[M+H]^+$, 313.26, 283.25	-	1.21	7.7	$1.0 \cdot 10^{-6}$	0.85
DAG(16:0/18:3)	591.48 $[M+H]^+$, 335.26, 313.26, 261.20	-	1.38	6.8	$1.0 \cdot 10^{-6}$	0.85
DAG(16:0/18:1)	595.48 $[M+H]^+$, 339.29, 313.27, 265.22, 239.22	-	1.17	6.1	$3.0 \cdot 10^{-6}$	0.84
DAG(18:3/18:3)	613.50 $[M+H]^+$, 335.24	-	1.20	2.3	$3.0 \cdot 10^{-6}$	0.82
DAG(18:3/18:2)	615.48 $[M+H]^+$, 337.27, 335.24, 263.20, 261.20	-	1.22	7.9	$2.5 \cdot 10^{-4}$	0.75
DAG(16:0/20:4)	617.50 $[M+H]^+$, 361.33, 313.27, 287.21	-	1.25	5.3	$1.5 \cdot 10^{-4}$	0.76
DAG(18:2/18:1)	619.51 $[M+H]^+$, 339.29, 337.24	-	1.26	3.2	$1.2 \cdot 10^{-4}$	0.75
Free fatty acids (FFA)						
Palmitoleic acid	-	253.22 $[M-H]^-$	1.49	5.1	$7.2 \cdot 10^{-4}$	0.81
Palmitic acid	-	255.22 $[M-H]^-$	1.36	10.6	$1.3 \cdot 10^{-3}$	0.78
Oleic acid	-	281.24 $[M-H]^-$	1.44	6.7	$5.4 \cdot 10^{-4}$	0.80
Low molecular weight metabolites						
Urea	61.04 $[M+H]^+$, 44.02	-	0.76	4.1	$9.9 \cdot 10^{-3}$	0.68
Alanine	90.06 $[M+H]^+$, 44.04	-	1.43	2.9	$1.0 \cdot 10^{-6}$	0.92
Taurine	-	124.00 $[M-H]^-$, 79.97	0.88	11.6	$2.7 \cdot 10^{-3}$	0.73
Picolinic acid	124.05 $[M+H]^+$, 106.02, 78.03	-	1.15	4.1	$4.1 \cdot 10^{-5}$	0.80
Creatine	-	130.06 $[M-H]^-$, 88.05	0.87	0.9	$4.9 \cdot 10^{-3}$	0.69
Malic acid	-	133.02 $[M-H]^-$, 115.03, 71.02	0.88	3.6	$9.7 \cdot 10^{-4}$	0.84
Dopamine	136.07 $[M+H-H_2O]^+$, 119.05, 91.04	-	0.77	3.3	$1.0 \cdot 10^{-6}$	0.93
Serotonin	177.09 $[M-H]^-$, 160.07, 115.05	-	0.90	3.3	$1.2 \cdot 10^{-2}$	0.89

Supplementary Material

Click here to download Supplementary Material: Supp Inf Table 1 - Demographic data of subjects enrolled in the study.docx

Dear Editor,

Please find enclosed the manuscript entitled "APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE" by R. Gonzalez Dominguez, T. Garcia barrera and J.L. Gómez Ariza.

The manuscript considers the use of atmospheric pressure photoionization (APPI) for metabolomic analysis of human serum sample. Atmospheric pressure photoionization (APPI) presents important advantages over other atmospheric pressure ionization sources, such as universality for the analysis of very different analytes, low susceptibility to matrix effects and high linear dynamic range. For these reasons, it is being introduced in metabolomic studies, normally hyphenated to liquid chromatography and capillary electrophoresis. However, the use of flow injection analysis for high throughput metabolomic screening has not been considered previously. Simultaneous analysis of both polar and non-polar compounds in APPI requires careful selection of working parameters, principally FIA solvent, dopant, and their flows. In addition, a suitable sample extraction procedure is crucial in metabolomics, together with the correct choice of MS conditions. In this work a novel procedure based on flow injection analysis atmospheric pressure photoionization mass spectrometry (FIA-APPI-Q-TOF-MS) is presented for metabolomic analysis of human serum samples. The use of complementary analytical methodologies combining a two-step serum extraction of polar and lipophilic metabolites with analysis by APPI(+)/APPI(-) ionization modes provided comprehensive metabolic profiles.

Yours sincerely,

José Luis Gómez Ariza.

APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE

Raúl González-Domínguez, Tamara García-Barrera, José Luis Gómez-Ariza

Novelty statement

This work describes for the first time the use of a metabolomic platform based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for the investigation of metabolic alterations associated with Alzheimer's disease.

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APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE

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The use of atmospheric pressure photoionization is not widespread in metabolomics, despite its considerable potential for the simultaneous analysis of compounds with diverse polarities. This work considers the development of a novel analytical approach based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for rapid metabolic screening of serum samples. Several experimental parameters were optimized, such as type of dopant, flow injection solvent, and their flows, given that a careful selection of these variables is mandatory for a comprehensive analysis of metabolites. Toluene and methanol were the most suitable dopant and flow injection solvent, respectively. Moreover, analysis in negative mode required higher solvent and dopant flows (100 µl min^{-1} and 40 µl min^{-1}, respectively) compared to positive mode (50 µl min^{-1} and 20 µl min^{-1}). Then, the optimized approach was used to elucidate metabolic alterations associated with Alzheimer's disease. Thereby, results confirm the increase of diacylglycerols, ceramides, ceramide-1-phosphate and free fatty acids, indicating membrane destabilization processes, and reduction of fatty acid amides and several neurotransmitters related to impairments in neuronal transmission, among others. Therefore, it could be concluded that this metabolomic tool presents a great potential for analysis of biological samples, considering its high-throughput screening capability, fast analysis and comprehensive metabolite coverage.

Keywords
Alzheimer's disease, metabolomics, atmospheric pressure photoionization, flow injection.

Novelty statement
This work describes for the first time the use of a metabolomic platform based on flow injection analysis and atmospheric pressure photoionization mass spectrometry for the investigation of metabolic alterations associated with Alzheimer's disease.

INTRODUCTION

The main challenge in metabolomics is to obtain comprehensive and unbiased metabolic fingerprints of samples due to the huge heterogeneity and dynamism of metabolome [1]. In this context, mass spectrometry represents a very interesting analytical platform, since complexity of metabolome may be overcome through the use of complementary atmospheric pressure ionization methods [2]. Electrospray (ESI) is the most common ionization source employed in metabolomic studies because it is able to ionize compounds in a wide range of masses and polarities, and it may be coupled to liquid chromatography [3-4], capillary electrophoresis [5-6] or used by direct infusion mass spectrometry [7]. A second alternative is atmospheric pressure chemical ionization (APCI), more suitable for less polar compounds [8-9]. Finally, atmospheric pressure photoionization (APPI) complements ESI and APCI for the analysis of little polar or non-polar compounds, but it has been considerably less used in metabolomics. Atmospheric pressure photoionization is the most recent soft ionization technique for mass spectrometry, introduced simultaneously by Bruins and Syage in 2000 [10-11], extending the range of ionizable compounds to less polar ones, which are not readily ionized by ESI and APCI. Nevertheless, APPI is capable of ionizing both polar and non-polar compounds through proton transfer and charge exchange reactions respectively, so it could be considered a universal ionization source [12]. The APPI interface uses a photoionization lamp and a dopant flow to form dopant radical ions, which can directly ionize non-polar analytes through charge exchange reactions. On the other hand, for polar compounds dopant photoions can produce intermediate reactive species by reactions with solvent or oxygen molecules, in positive and negative ionization modes respectively, followed by a proton transfer reaction to the analyte [12]. Thereby, for the simultaneous analysis of both polar and non-polar compounds, the two reaction pathways must be accessible, which requires a careful selection of solvent, dopant, and their flows. Firstly, the carrier solvent employed can lead to a preferential mechanism, being favored charge exchange for low proton affinity solvents, whereas the addition of methanol or acetonitrile initiates proton transfer [13]. In the case of dopant, the most common reagent is toluene, but it may be not suitable for the ionization of non polar compounds in high proton affinity solvents since tends to transfer its proton to the solvent [14]. Alternatively, other dopants less reactive with the solvent have been proposed, such as anisole [15] or substituted benzenes [16]. Finally, the flow-rate of both solvent and dopant also play important roles in the photoionization process. In the case of solvent, ionization efficiency decreases when the flow increases, due to the formation of large unreactive protonated-solvent cluster ions, while signal increases with the dopant flow, until reach 5-10% of solvent flow [17]. Moreover, it has been reported that toluene can provide high ionization efficiency simultaneously to both polar and non-polar compounds delivered in reversed-phase solvents, simply limiting the solvent flow rate in order to avoid that reactions between toluene photoions and solvent were driven to completion [18].

In this way, APPI might present a considerable potential in metabolomics, not only because of its universality in terms of ionization capability, but also because is less susceptible to matrix effects and presents a linear dynamic range, generally higher than that for ESI [19-20]. In addition, it requires less heat for desolvation than APCI allowing analysis of thermally labile compounds [21]. APPI has been used for the analysis of many classes of compounds, including pharmaceutical drugs and metabolites [22], steroids [21], aldehydes and ketones [23] or pesticides [24]. However, only a few non targeted approaches include APPI as an alternative in metabolomics. Thereby, metabolomics based on liquid chromatography mass spectrometry and the integration of multiple ionization modes (ESI, APCI and APPI) has been previously proposed for the study of urine [25] and plasma samples [26], or to perform a more comprehensive analysis of lipidome [27-28]. Although APPI is normally coupled to liquid chromatography [29] or capillary electrophoresis [30], several reports confirm the ability of flow injection analysis for the determination of fullerenes and perfluorinated compounds [31], characterization of wine [32], olive oil [33] or Iberian ham [34], or the study of drugs [35] and petroleum [36]. This high-throughput approach exhibits several advantages in metabolomics such as fast and reproducible analysis, and comprehensive non-targeted metabolite coverage [7]. In addition, the use of high resolution systems such as time-of-flight (TOF) or Fourier transform ion cyclotron resonance (FTICR) allows overcoming problems associated with isobaric interferences [37]. However, to date, the use of FIA-APPI-MS has not been considered in metabolomic analysis.

This work explores for the first time the potential of flow injection analysis and high resolution tandem mass spectrometry with atmospheric pressure photoionization source (FIA-APPI-QTOFMS) in metabolomics. Several critical parameters were optimized for the simultaneous analysis of both polar and non-polar compounds in positive and negative ionization modes, such as type of dopant, flow injection solvent and their working flows. For this purpose, a test mixture of representative metabolites from human serum was used. Then, the optimized approach was applied to blood serum samples in order to investigate metabolic abnormalities associated with Alzheimer's disease (AD). Multivariate statistics demonstrated the ability of this high-throughput metabolomic tool for

discriminating between AD patients and healthy controls, and allowed the identification of different metabolic failures underlying to pathological features of this health disorder.

MATERIAL AND METHODS

CHEMICALS AND SAMPLES

The solvents (HPLC-grade) methanol, ethanol, chloroform, dichlorometane, acetonitrile and isopropanol were purchased from Fisher Scientific (Leicestershire, UK), while dopants toluene, anisole and chlorobenzene were supplied by Aldrich (Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Standards of L-glutamine, L-valine, L-cysteine, L-aspartic acid, L-arginine, L-histidine, L-glutamic acid, L-phenylalanine, D-glucose, creatine, creatinine, cholesterol, di-oleoyl-phosphocholine and triolein were from Sigma Aldrich. Blood samples were obtained by puncture of the antecubital vein and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. The samples were immediately cooled and protected from light for 30 minutes, and after centrifugation (3500 rpm for 10 minutes) serum was aliquoted and frozen at -80°C until analysis. Alzheimer's disease patients (AD, N=30, age 78.3±5.0, male/female 12/18) were newly diagnosed of sporadic Alzheimer's disease by the Neurologic Service of Hospital Juan Ramón Jiménez (Huelva, Spain), according to the criteria of the NINCDS-ADRDA [38]. Healthy controls (HC, N=30, age 73.5±5.9, male/female 10/20), who had not more than two reported cases of Alzheimer's disease in their families, were studied by neurologists to confirm the absence of neurological disorders. The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of University of Huelva.

PREPARATION OF SAMPLES

Optimization of experimental conditions was performed with a solution containing standards of representative metabolites from blood serum in methanol:water (1:1, v/v), including different low molecular weight compounds from different chemical classes and lipids, ranging diverse acid-base properties and polarities. The composition of this mixture was selected according to their usual concentrations in human serum described in the Human Metabolome Database (HMDB), as shown in Table 1. This metabolomic approach was further tested in real serum, for which samples were treated in a two-stage sequential procedure described elsewhere for comprehensive serum metabolomics [39]. For the extraction of metabolites, 100 µL of serum are mixed with 400 µL of methanol/ethanol 1:1 and stirred for 5 min, followed by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant is transferred to another tube, and the precipitate is kept for further treatment. Then, supernatant is dried under nitrogen stream and the resulting residue reconstituted with 80 µL of methanol and 20 µL of water (polar extract). On the other hand, the precipitate isolated in the first step is extracted with 400 µL of chloroform:methanol1:1 by stirring during 5 min, followed by centrifugation at 10000 rpm for 10 minutes at 4 °C. Finally, the resulting supernatant is taken to dryness under nitrogen stream and reconstituted with 100 microliters of 60:40 dichloromethane:methanol (lipophilic extract).

INSTRUMENTATION

Mass spectrometry experiments were performed on a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure photoionization (APPI) source. Samples were introduced by flow injection using an Accela LC system (Thermo Fisher Scientific) equipped with autosampler and quaternary pump. In addition, a model KDS 100 syringe pump from KD scientific (New Hope, PA, USA) was employed to deliver the dopant for photospray ionization. Data were obtained both in positive and negative ion modes, injecting 10 µl of sample, and acquiring full scan spectra in the m/z range 50-1100 with 1.005 seconds of scan time. Acquisition time was selected according to the LC flow employed to consider the dispersion of the plug of sample into the solvent stream, and employing some delay time between injection and analysis to allow enough time for the sample to reach the mass spectrometer (settings shown in Table 2). The ion spray voltage (IS) was set at 1500 V and -2300 V in positive and negative modes respectively, with declustering potential (DP) of ±50 V and focusing potential (FP) of ±250 V. The source temperature was maintained at 400ºC, and the gas flows (high-purity nitrogen) were fixed at 1.13 L min^{-1} for curtain gas, 1.50 L min^{-1} for nebulizer gas, 3.0 L min^{-1} for heater gas, and 1 L min^{-1} for lamp gas. The optimization of FIA conditions was performed in four steps: type of dopant, type of carrier solvent, solvent flow and, finally, dopant flow. For the analysis of serum samples, the optimal dopant was toluene delivered at 20/40 µl min^{-1}, in positive and negative ion mode respectively. Furthermore, methanol was used as flow injection solvent at 50/100 µl min^{-1}, in both ionization modes.

STATISTICAL ANALYSIS

Metabolomic data were submitted to peak detection by Markerview™ software (Applied Biosystems) in order to filter the mass spectrometry results, and to carry out the reduction into a two-dimensional data matrix of spectral

peaks and their intensities. For this, the peak search was done with a mass tolerance of 0.1Da, and a minimum response of 10 counts was considered for filtering. Then, data was processed in SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden) in order to find differences between the groups of study (AD patients and healthy controls). Partial least squares discriminant analysis (PLS-DA) was performed to build predictive models that provide class separation and allow the further study of discriminant metabolites. Quality of the models was assessed by the R^2 and Q^2 values, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). Finally, discriminant compounds were selected according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 2.0, indicative of significant differences among groups.

IDENTIFICATION OF METABOLITES

Discriminant metabolites were identified matching the experimental accurate mass and tandem mass spectra (MS/MS) with those available in metabolomic databases (HMDB, METLIN, LIPIDMAPS), using a mass accuracy of 50 ppm. Then, different classes of lipids were confirmed based on characteristic fragmentation patterns previously described. It is noteworthy that signals from APPI mass spectra can be mainly attributed to protonated or deprotonated adducts (in positive and negative modes, respectively), but the possibility to produce dehydrated molecular ions has been also previously demonstrated [40-42]. Ceramides presented characteristic product ions in positive ionization mode at m/z 264 and 282 due to the fragmentation in the sphingosine moiety [43]. On the other hand, fragmentation of glycerolipids (diacylglycerols) occurs through the release of fatty acids generating different types of ions (named A, B, C and D), which show characteristic m/z values according to the fatty acid attached to the glycerol backbone [34]. Finally, fatty acid amides were confirmed with characteristic fragments described in the literature [44].

RESULTS AND DISCUSSION

SELECTION OF EXPERIMENTAL CONDITIONS

The effect of experimental conditions on the photoionization efficiency was evaluated by flow injection analysis of a mixture solution test of representative metabolites (Table 1), acquiring in both positive and negative ion modes. Firstly, for the selection of dopant the other parameters were adjusted according to previous studies for the simultaneous determination of both polar and non-polar compounds [18], employing a mixture of methanol:water (1:1, v/v) as solvent at a flow rate of 100 µl min^{-1}. Dopant flow was fixed at a 10% of solvent flow (10 µl min^{-1}), since a plateau in ionization efficiency is reached in this source as more dopant is added [17]. The most common dopants described in literature were tested in this study (i.e. toluene, chlorobenzene and anisole), and the ionization efficiencies were compared in function of peak intensities (Fig. 1). In positive ionization mode (Fig. 1a) the best sensitivity was obtained with toluene for most of the standards, followed by chlorobenzene (it should be noted that the great increase in intensity observed for histidine with chlorobenzene is attributable to the presence of an impurity in this solvent with the same mass, which was checked using a blank test). However, for lipid compounds the peak intensity is slightly increased with anisole and chlorobenzene, although none of these dopants allowed the ionization of di-oleoyl-phosphocholine. On the other hand, intensities of low molecular weight metabolites were very similar in negative mode using the three dopants, although generally lower than that in positive mode, while lipids were not detected. The enhanced ionization observed with toluene and chlorobenzene compared to anisole could be due to their higher ionization energy (IE) values (8.83, 9.07, and 8.20 eV, respectively), given that for an effective photoionization the IE of the dopant must be higher than that of the analytes. Moreover, the better sensitivity of toluene compared to chlorobenzene for polar metabolites in positive mode, despite its lower IE, may be due to the impossibility of chlorobenzene to perform photoinozation by proton transfer [16]. Conversely, this low reactivity of chlorobenzene and anisole with the solvent is also responsible for the increased signal of non-polar analytes such as cholesterol and triolein using these dopants, although good results were also obtained with toluene. Therefore, toluene was selected as dopant for further experiences, since it provides acceptable results with most of metabolites with the highest mean sensitivity. In a second step, different solvents were evaluated for the analysis of metabolites by FIA-APPI-MS (Fig. 2). Besides the mixture methanol:water 1:1, other solvents usually employed in liquid chromatography mass spectrometry such as methanol and acetonitrile were tested, as well as isopropanol, a photoionizable solvent that may initiates ionization of analytes [27,40]. The solvent was delivered at a flow rate of 100 µl min^{-1}, and mixed with toluene at 10 µl min^{-1}. Compared with methanol:water, the use of pure methanol slightly increased sensitivity, according to previous works in which a reduction of the water content produced a general increase of sensitivity [45]. However, acetonitrile reduced the photoionization efficiency respect to

methanol, probably due to more effective nebulization and vaporization processes provided by methanol [22] and the higher proton affinity (PA) of acetonitrile [24]. Finally, isopropanol produced comparable results to methanol, so it could be concluded that is not necessary to use photoionizable solvents if experimental conditions are well selected. The solvent flow is other important factor to be considered when the APPI source is employed, as it has been previously revised [17,46-47]. Using methanol as solvent and toluene as dopant, solvent flow was ranged between 50-500 µl min^{-1} maintaining the dopant flow at a 10%. The monitoring of the intensity for three representative standards depending on the flow is represented in Figure 3, which shows a different tendency when results from positive and negative ionization modes are compared. In positive mode, the maximum intensity was reached at low flow rates (about 50 µl min^{-1}), decreasing progressively when solvent flow increases. The loss of sensitivity at higher solvent flow rates can be attributed to different processes, such as the growth of ion-solvent clusters, because larger clusters may be unreactive and stimulate the loss of ions by recombination processes [17]. Furthermore, charge exchange is reduced due to the loss of dopant radical cations by reaction with the solvent molecules [46]. On the other hand, the highest ionization efficiency in negative mode is achieved with a solvent flow of 100 µl min^{-1} (Fig. 3b). Negative ionization is much less used in APPI, and the effect of solvent flow in photoionization has not been previously considered. However, negative ionization takes place through different mechanisms, such as electron capture, charge exchange, proton transfer, or substitution reactions [48-49], so it is not rare to find a different behavior according to the flow of solvent compared with positive mode. Finally, dopant flow was tested around 10-50% of the solvent flow previously selected. Thus, in positive ionization mode the dopant flow was ranged between 5-25 µl min^{-1}, but higher values were employed in negative mode, in the range of 10-50 µl min^{-1} (Fig. 4). Results show that ionization efficiency reaches a plateau at high dopant flows (around 30-40% of solvent flow) in both analysis modes. This finding is consistent with data presented by other authors for low flow applications, as is the case of the present study. Thereby, although under conventional LC conditions ionization efficiency quickly remains constant as more dopant is added, at about 5-10% of the solvent flow [17], it has been observed that this may result in a low rate of reagent ion production when solvent flow is reduced, so that the addition of more dopant may be advantageous [12]. Therefore, optimal dopant flows were set at 20 and 40 µl min^{-1}, for positive and negative modes of analysis, respectively.

APPLICATION OF FIA-APPI-MS TO A METABOLOMIC STUDY OF ALZHEIMER'S DISEASE

The suitability of the optimized APPI approach for the analysis of blood serum samples was confirmed in a metabolomic investigation of Alzheimer's disease. The potential of a two-steps methodology for serum extraction, complemented by the use of positive and negative ionization modes has been previously demonstrated for comprehensive metabolomic fingerprinting of serum samples by direct infusion electrospray mass spectrometry [39]. Similarly, analysis by FIA-APPI-MS of these serum extracts generated mass spectra with a high diversity of signals, as shown in Figure 5. In this sense, it is noteworthy that for polar extracts the most prominent signals are observed at low m/z values, below 500 amu (Figs. 5a1 and 5a2), while in lipophilic extracts several clusters of peaks are obtained in the m/z range 500-1000 (Figs. 5b1 and 5b2), allowing a comprehensive assessment of serum metabolites. Then, results were submitted to partial least squares discriminant analysis (PLS-DA) in order to discriminate between AD patients and healthy controls. The figure 6 shows the scores plots of all the models, for APPI(+)-MS and APPI(-)-MS data from polar and lipophilic extracts, which provides a good classification of samples. Furthermore, the parameters R^2 and Q^2, listed in Table 3, were used to evaluate the performance of these models. As can be observed, satisfactory values for the quality parameters R^2 and Q^2 were obtained, with a variance explained around 100% and variance predicted above 80%, demonstrating the potential of flow injection analysis with APPI-MS for serum metabolomics. Finally, metabolites responsible for discrimination were selected according to the Variable Importance in the Projection parameter (VIP>2). These discriminant metabolites are listed in Table 4, including several low molecular weight metabolites and different lipid classes.

METABOLIC ALTERATIONS IN SERUM FROM ALZHEIMER'S DISEASE

Metabolic fingerprints have shown important differences in serum from AD patients respect to healthy controls, considering up- or down-regulation of metabolites belonging to diverse classes, from lipids such as fatty acid or ceramides to amino acids and neurotransmitters. After identification of altered metabolites, it is important to decipher disturbed cellular pathways in order to understand the biochemical processes underlying to disease. Thus, a tentative biochemical interpretation of results is presented in the next paragraphs.

Fatty acid amides and the endocannabinoid system

Primary fatty acid amides (PFAM) are bioactive lipids found in several tissues and biological fluids of mammals, including humans [44,50]. These compounds are important endogenous signaling molecules in the mammalian

nervous system, binding to many receptors and demonstrating control over a variety of biological processes such as sleep regulation, modulation of monoaminergic systems, locomotion, inhibition of phospholipase A_2 (PLA_2) and epoxide hydrolase (EH), and many other processes [51-52]. Moreover, although these lipids do not interact with cannabinoid receptors, they are catabolically related to endocannabinoids and might regulate biological activities in mammals in a similar fashion [53]. Furthermore, PFAMs are also degraded by fatty acid amide hydrolase (FAAH) [54], the principal catabolic enzyme of the endocannabinoid system. In the context of Alzheimer's disease, there is growing evidence for the involvement of the endocannabinoid system in the development of neurodegeneration [55]. Thereby, the expression levels of cannabinoid receptors (CB) have been found altered in AD brains, including a decrease of CB1 receptors and up-regulation of CB2 receptors in the hippocampus [56]. In addition, a considerable over-expression of FAAH activity has been also associated with AD [57]. Therefore, the decrease of different fatty acid amides observed in this work (palmitoleamide, palmitamide, linolenamide, linoleamide, oleamide and stearamide) could be considered as an indirect marker of endocannabinoid dysregulation in Alzheimer's disease, as previously reported for serum oleamide [58].

Alterations in sphingolipid metabolism: increase of ceramides

The increase of serum ceramides (CER) and ceramide-1-phosphate (C1P) shown in Table 4 is consistent with impaired sphingolipid metabolism, whose involvement in AD has been extensively discussed [59]. The proper balance of sphingolipids is essential for neuronal function, given that sphingomyelins (SM) are critical components of lipid membrane rafts, and their catabolites are bioactive compounds and second messengers in cellular signaling and apoptosis. The sphingolipid pathway is altered at the gene expression level in AD, with up-regulated activities of enzymes controlling ceramide synthesis [60] and acid sphingomyelinase [61], suggesting a shift in metabolism towards the accumulation of ceramides. Moreover, the contribution of ceramides to AD pathogenesis has been proposed on the basis of their activity as second messengers, being directly involved in apoptotic signaling, controlling secretion of APP and Aβ, or stimulating PLA_2 [62].Thereby, high levels of total ceramides have been previously associated with AD, mainly in post-mortem studies of brain tissue [60-61,63], but also in cerebrospinal fluid [64] and once in plasma [65], in agreement with our metabolomic findings.

Diacylglycerols and fatty acids: the role of phospholipases

The accumulation of diacylglycerols (DAGs) and free fatty acids (FFAs) in serum from AD patients (Table 4) has been previously associated with cellular membrane breakdown due to over-activation of phospholipases [58]. Abnormalities in membrane phospholipids in AD are principally related to altered expression of phospholipase A_2 (PLA_2) function [66], but there is also evidence for the role of phospholipases C and D, although they has been much less studied [67-68]. Furthermore, it is known the interconnections between the metabolism of phospholipids and sphingomyelins [69]. Ceramides and ceramide-1-phosphate induce overactivation of PLA_2, as stated in the previous section, while arachidonic acid (one of the most abundant fatty acids contained in neural phospholipids) released from the hydrolysis of these phospholipids by the action PLA_2, causes the overexpression of sphingomyelinase (SMase). Therefore, it could be concluded that membrane breakdown processes in Alzheimer's disease are finally reflected in multiple inter-related metabolites that are altered in serum samples, as schematized in Figure 7.

Involvement of low molecular weight metabolites

The involvement of low molecular weight metabolites in central pathways of metabolism makes them important targets in the study of pathological alterations underlying to diseases. In this study, the most important changes could be related to disturbances in neurotransmitter systems, given that decreased serum levels of serotonin, dopamine and taurine were found (Table 4). Serotonin and dopamine are two important monoaminergic neurotransmitters, whose altered synthesis have been previously associated with synaptic failure leading to neurodegeneration in Alzheimer's disease [70]. On the other hand, taurine presents several roles in neurotransmission, neuromodulation, osmoregulation, control of calcium influx and cell excitability, whose implication in neurodegeneration and AD has been previously demonstrated [71]. Hypometabolism is also documented in AD, with depleted neuronal energy production by decreased rate of carbohydrate catabolism [72] and oxidative deficiency characterized by mitochondrial dysfunction [73]. This energetic deficiency is in accordance with the altered levels of alanine, creatine and malate observed in metabolomic profiles (Table 4), given that these compounds participate in overall cellular bioenergetics. Picolinic acid is a tryptophan metabolite from the kynurenine pathway, which is up-regulated in AD by over-expression of indole-2,4-dioxygenase. Thereby, altered synthesis of related neuroactive compounds and the accumulation of different derivatives have been previously reported in AD [74], in agreement with the increased levels of picolinic acid observed in this study. Finally, the

reduction of urea levels points to a perturbation of the urea cycle, responsible for controlling ammonia concentrations in the organism. The alteration of this pathway in AD has been previously demonstrated on the basis of altered levels of expression in different enzymes and the corresponding genes [75], which finally results in altered content of related metabolites as found in our metabolomic study.

CONCLUSIONS

A high throughput approach based on flow injection analysis and high resolution tandem mass spectrometry with atmospheric pressure photoionization source (FIA-APPI-QTOFMS) has been developed for metabolomic analysis of serum samples. Several experimental parameters affected considerably the ionization efficiency, such as dopant, carrier solvent or the flow rate of both. Toluene and methanol were selected as optimal dopant and flow injection solvent respectively, providing the highest sensitivity for the analysis of most of the metabolites. Considering the delivery speed of solvent and dopant, different behavior was observed for positive and negative analysis modes. In negative mode, the ionization was favored at higher solvent and dopant flows (100 µl min^{-1} and 40 µl min^{-1}), while in positive mode lower flows are recommended (50 µl min^{-1} and 20 µl min^{-1}). Then, the suitability of this APPI-MS technique was demonstrated in a metabolomic investigation of Alzheimer's disease. Thereby, eight low molecular weight metabolites and numerous lipids belonging to four different classes were detected as potential markers of disease. Altered levels of primary fatty acid amides and major neurotransmitters such as serotonin or dopamine could be related to severe failures in neurotransmission. Moreover, important impairments associated with cellular membrane destabilization were also found, considering increased serum levels of ceramides, diacylglicerols and free fatty acids. Furthermore, other metabolic changes may be related to hypometabolism, hyperammonemia or up-regulation of the kynurenine pathway associated with this disease.

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FIGURE CAPTIONS

Figure 1. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different dopants.
Figure 2. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different solvents.
Figure 3. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different solvent flows.
Figure 4. Average peak intensities (n=3) of metabolites in positive ion mode (a) and negative ion mode (b) using different dopant flows.
Figure 5. Mass spectra from serum samples for APPI+ (1) and APPI- (2), with polar (a) and lipophilic (b) extracts.
Figure 6. Scores plots of the PLS-DA for APPI+ (1) and APPI– (2), with polar (a) and lipophilic (b) extracts. Black squares: females; red circles: males.
Figure 7. Membrane degradation and interconnections between metabolism of phospholipids and sphingolipids. ↑: compounds up-regulated, ↓: compounds down-regulated, dashed arrows: enzymatic stimulation

Table 1. Composition of metabolites standard test mixture

Compound	**Concentration (µM)**
Creatinine (Crn)	60
Valine (Val)	230
Cysteine (Cys)	200
Creatine (Cr)	50
Aspartic acid (Asp)	20
Glutamine (Gln)	600
Glutamic acid (Glu)	50
Histidine (His)	90
Phenylalanine (Phe)	70
Arginine (Arg)	100
Glucose (Glc)	5000
Cholesterol (Chol)	5000
Di-oleoyl-phosphocholine (PC)	5
Triolein (TG)	30

Table 2. Acquisition time parameters according to the LC flow

LC flow (µl min^{-1})	**Delay time (sec)**	**Acquisition time (min)**
50	20	0.7
100	12	0.5
200	5	0.4
300	3	0.3
500	0	0.2

Table 3. Statistical parameters of the PLS-DA models

ion mode	extract	R^2	Q^2
APPI+	polar	0.998	0.849
	Lipophilic	0.997	0.859
APPI-	Polar	0.999	0.873
	lipophilic	1	0.883

Table 4. Discriminant metabolites identified in serum from Alzheimer's disease patients

compound	m/z		fold change
	APPI+	APPI-	
Fatty acid amides (PFAM)			
Palmitoleamide	254.22 $[M+H]^+$	-	0.84
Palmitamide	256.24 $[M+H]^+$	-	0.84
Linolenamide	278.22 $[M+H]^+$	-	0.82
Linoleamide	280.25 $[M+H]^+$	-	0.76
Oleamide	282.26 $[M+H]^+$	-	0.72
Stearamide	284.27 $[M+H]^+$	-	0.75
Ceramides (CER)			
CER(d18:1/C16:1)	518.48 $[M+H-H_2O]^+$	516.48 $[M-H-H_2O]^-$	1.24
CER(d18:1/C16:0)	520.50 $[M+H-H_2O]^+$	518.49 $[M-H-H_2O]^-$	1.28
CER(d18:1/C18:1)	546.49 $[M+H-H_2O]^+$	-	1.16
CER(d18:1/C18:0)	-	546.46 $[M-H-H_2O]^-$	1.56
CER(d18:1/C18:0)-1-phosphate	628.57 $[M+H-H_2O]^+$	626.56 $[M-H-H_2O]^-$	1.33
CER(d18:1/C24:1)	630.60 $[M+H-H_2O]^+$	-	1.39
Diacylglycerols (DAG)			
DAG(C14:1/C16:0)	539.42 $[M+H]^+$	-	1.21
DAG(C16:0/C18:3)	591.48 $[M+H]^+$	-	1.38
DAG(C16:0/C18:1)	595.48 $[M+H]^+$	-	1.17
DAG(C18:3/C18:3)	613.50 $[M+H]^+$	-	1.20
DAG(C18:3/C18:2)	615.48 $[M+H]^+$	-	1.22
DAG(C16:0/C20:4)	617.50 $[M+H]^+$	-	1.25
DAG(C18:2/C18:1)	619.51 $[M+H]^+$	-	1.26
Free fatty acids (FFA)			
Palmitoleic acid	-	253.22 $[M-H]^-$	1.49
Palmitic acid	-	255.22 $[M-H]^-$	1.36
Oleic acid	-	281.24 $[M-H]^-$	1.44
Low molecular weight metabolites			
Urea	61.04 $[M+H]^+$	-	0.76
Alanine	90.06 $[M+H]^+$	-	1.43
Taurine	-	124.00 $[M-H]^-$	0.88
Picolinic acid	124.05 $[M+H]^+$	-	1.15
Creatine	-	130.08 $[M-H]^-$	0.87
Malic acid	-	133.02 $[M-H]^-$	0.88
Dopamine	136.07 $[M+H-H_2O]^+$	-	0.77
Serotonin	-	175.08 $[M-H]^-$	0.90

APPLICATION OF A NOVEL METABOLOMIC APPROACH BASED ON ATMOSPHERIC PRESSURE PHOTOIONIZATION MASS SPECTROMETRY USING FLOW INJECTION ANALYSIS FOR THE STUDY OF ALZHEIMER'S DISEASE

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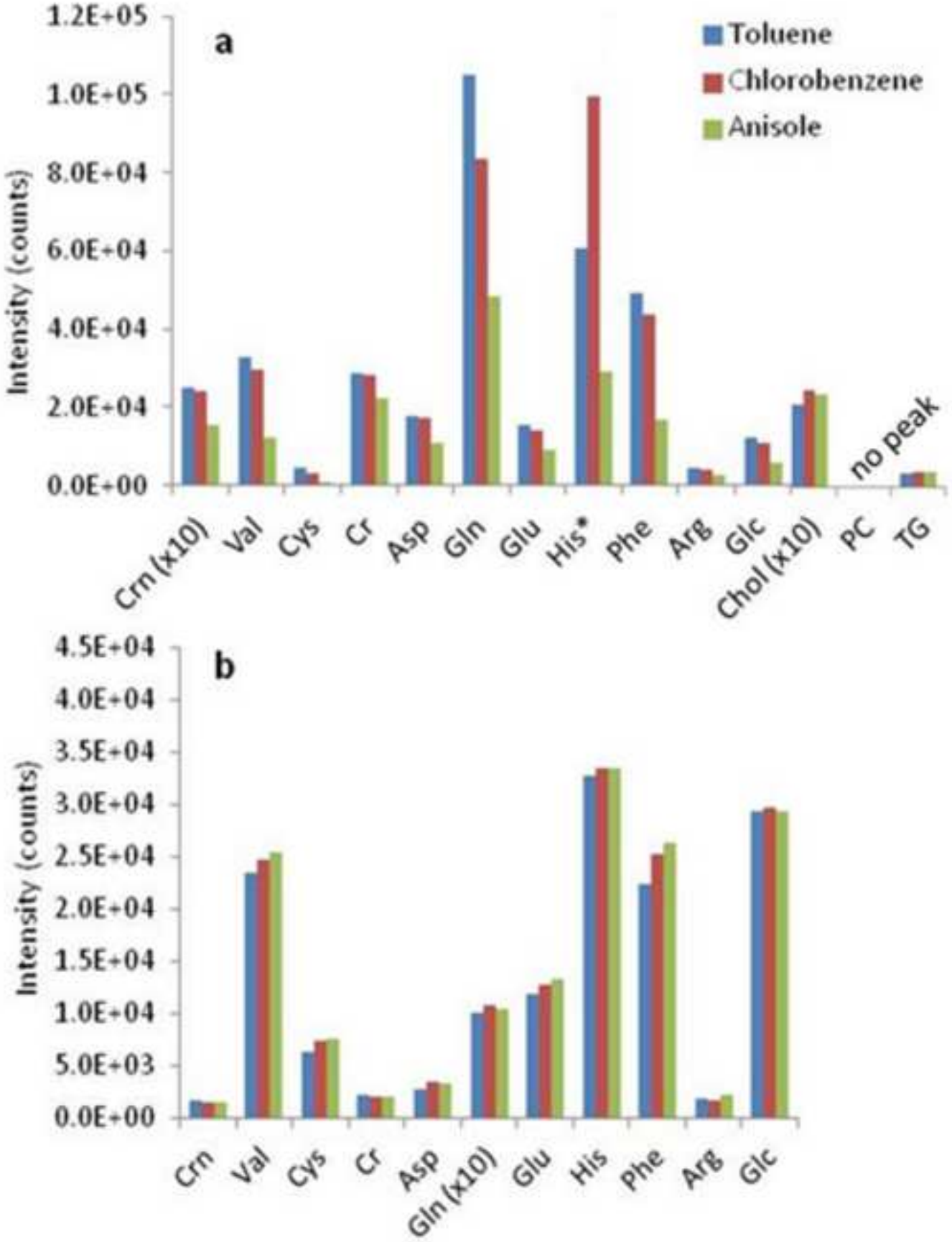

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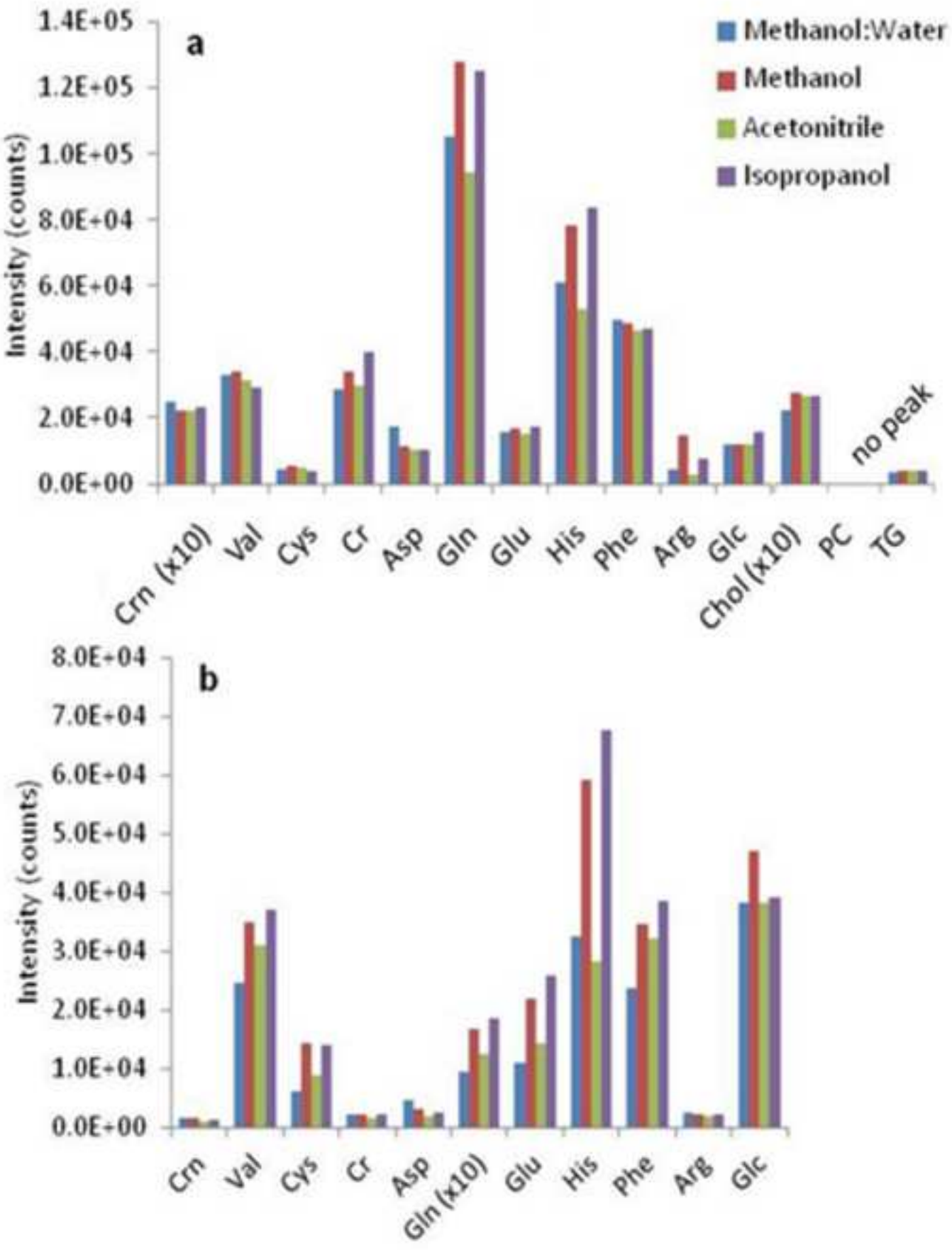

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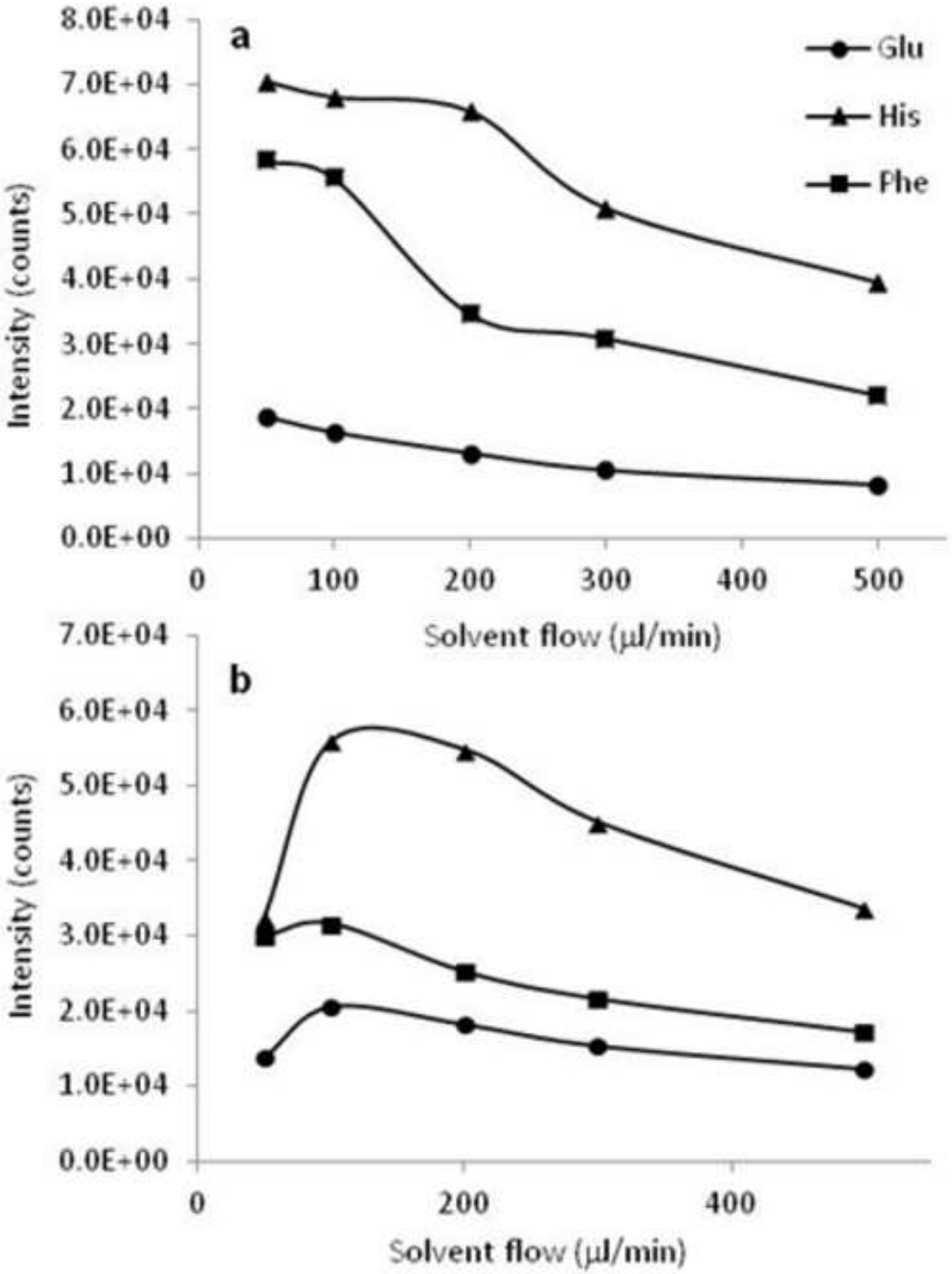

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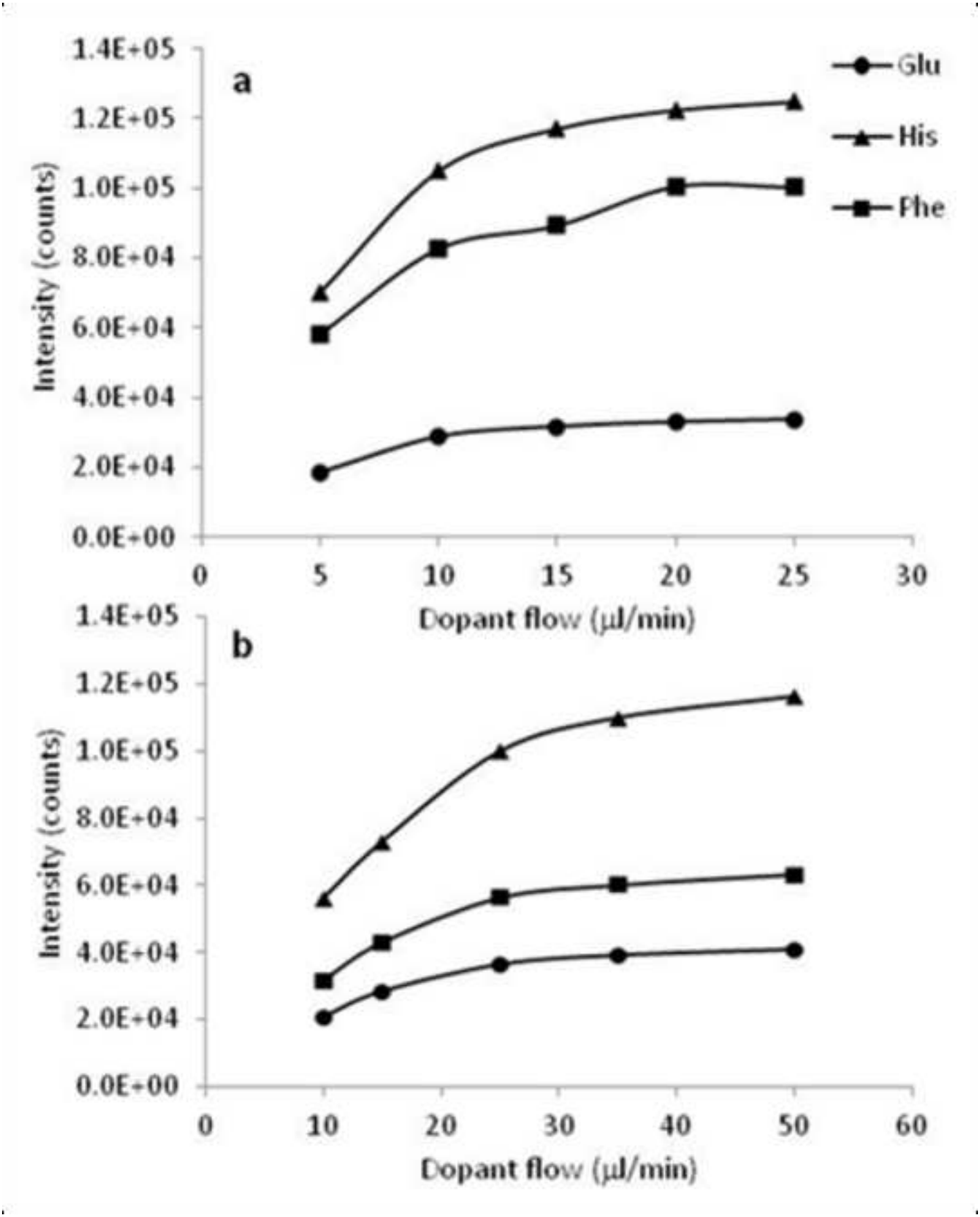

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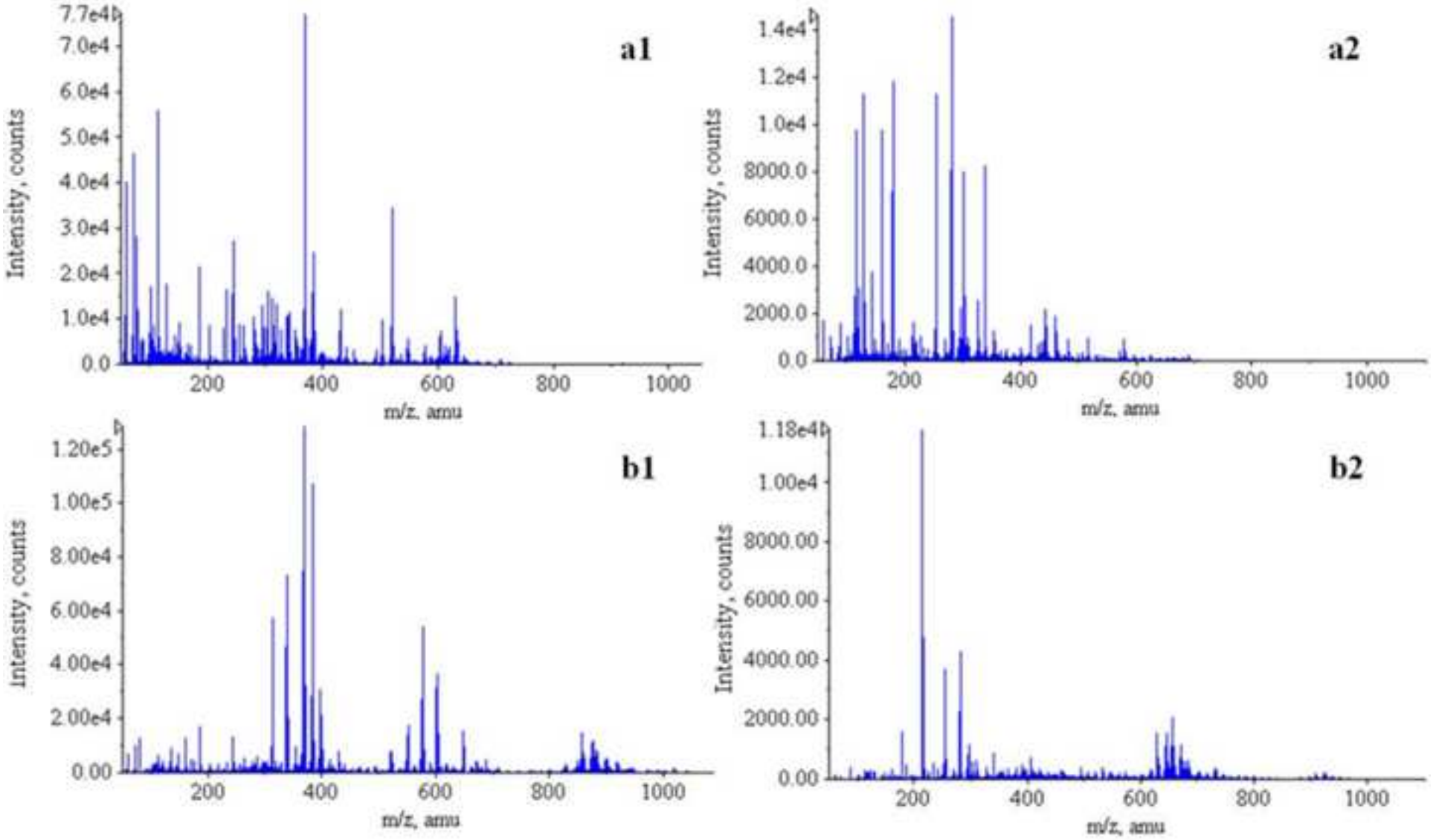

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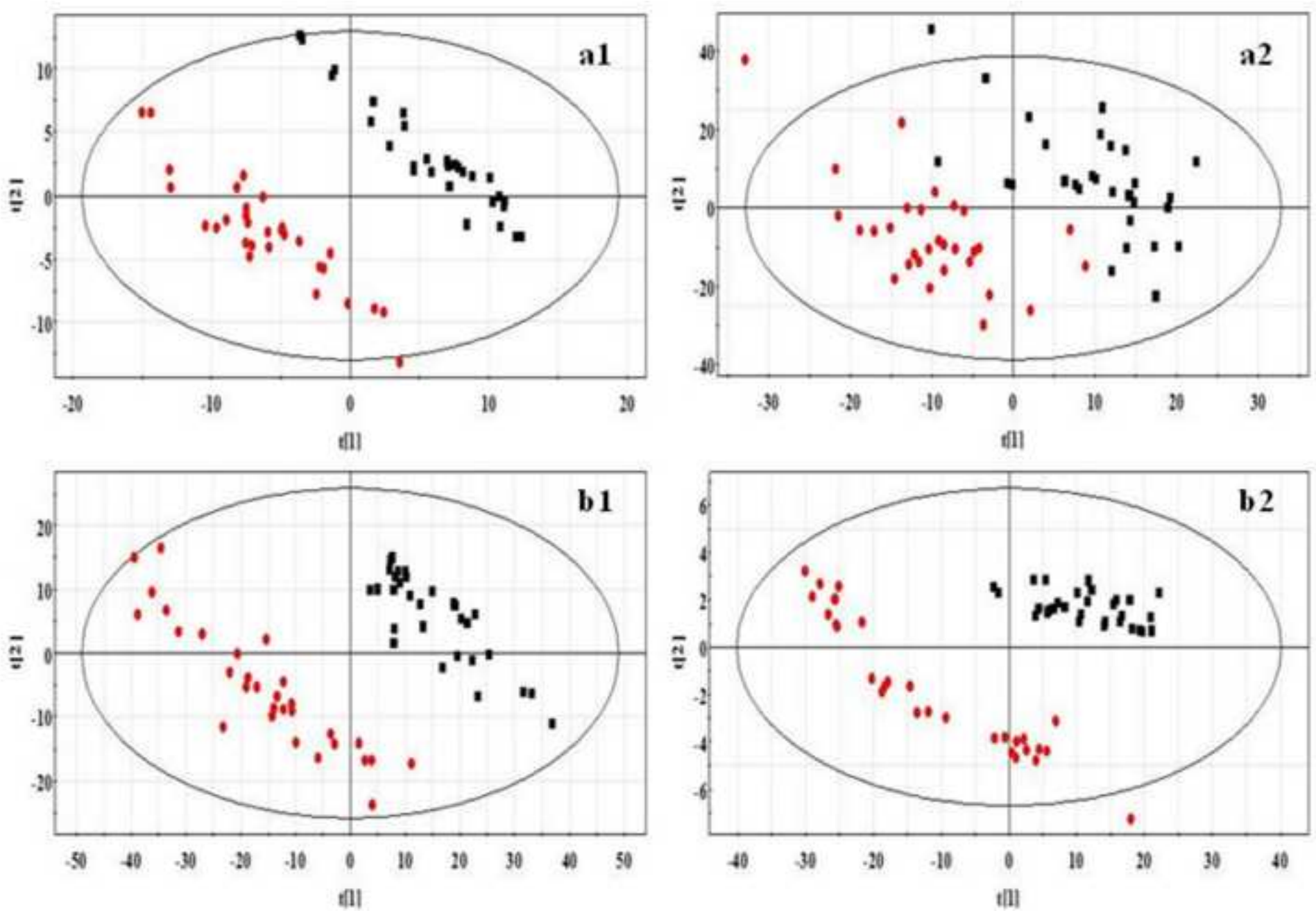

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