SCIENTIFIC REPORT OF THE SHORT TERM SCIENTIFIC MISSION (STSM)

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CONFIRMATION OF THE PRESENCE OF ACETYLCYSTEINYL PHENOLIC METABOLITES IN HUMAN CELLS MEDIA

STSM dates: from 29-02-2016 to 11-03-2016
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Objective

The main objective of this STSM was to perform an analysis by mass spectrometry of ultrahigh resolution associated with nuclear magnetic resonance integrated with in-line solid phase extraction (LC-MS-SPE-NMR) in samples obtained in cell assays with human brain endothelial cells where the ID of the same compounds were suggested.

Introduction

Beneficial effects resulting from berries consumption has been reported by several authors. Such studies have also been validated by intervention studies, concerning the elucidation about bioavailability of polyphenols present in berries and identification of their metabolites circulating in the blood. A previous study in human volunteers revealed the presence of acetylcysteinyl metabolites in urine and plasma. The absence of standard compounds and/or technique that define clearly the structure of the metabolites hampered the confirmation of the identity only based in exact mass and MS fragmentation.

This STSM was important for the confirmation of the presence of these compounds in human biological samples and to be the starting point for a confirmatory human trial for the presence of acetylcysteinyl phenolic derivatives in humans and its interindividual variability.

Results, discussion and conclusions

The samples from the studies performed in iBET were analysed as described.

The samples were dissolved in two different solvents, MeOH and 5% (vol/vol) acetonitrile in 0.1% (vol/vol) formic acid and the results obtained in both samples were the same. The identification of phenolic metabolites was made by comparison with the retention time and exact mass obtained in a previous study. The major peaks identified were ascorbic acid, taxifolin (internal standard) and the compound added in the cells (pyrogallol-sulfate), the acetylcysteine metabolite, glutathionyl metabolites and others were not possible to identify (Fig 1).

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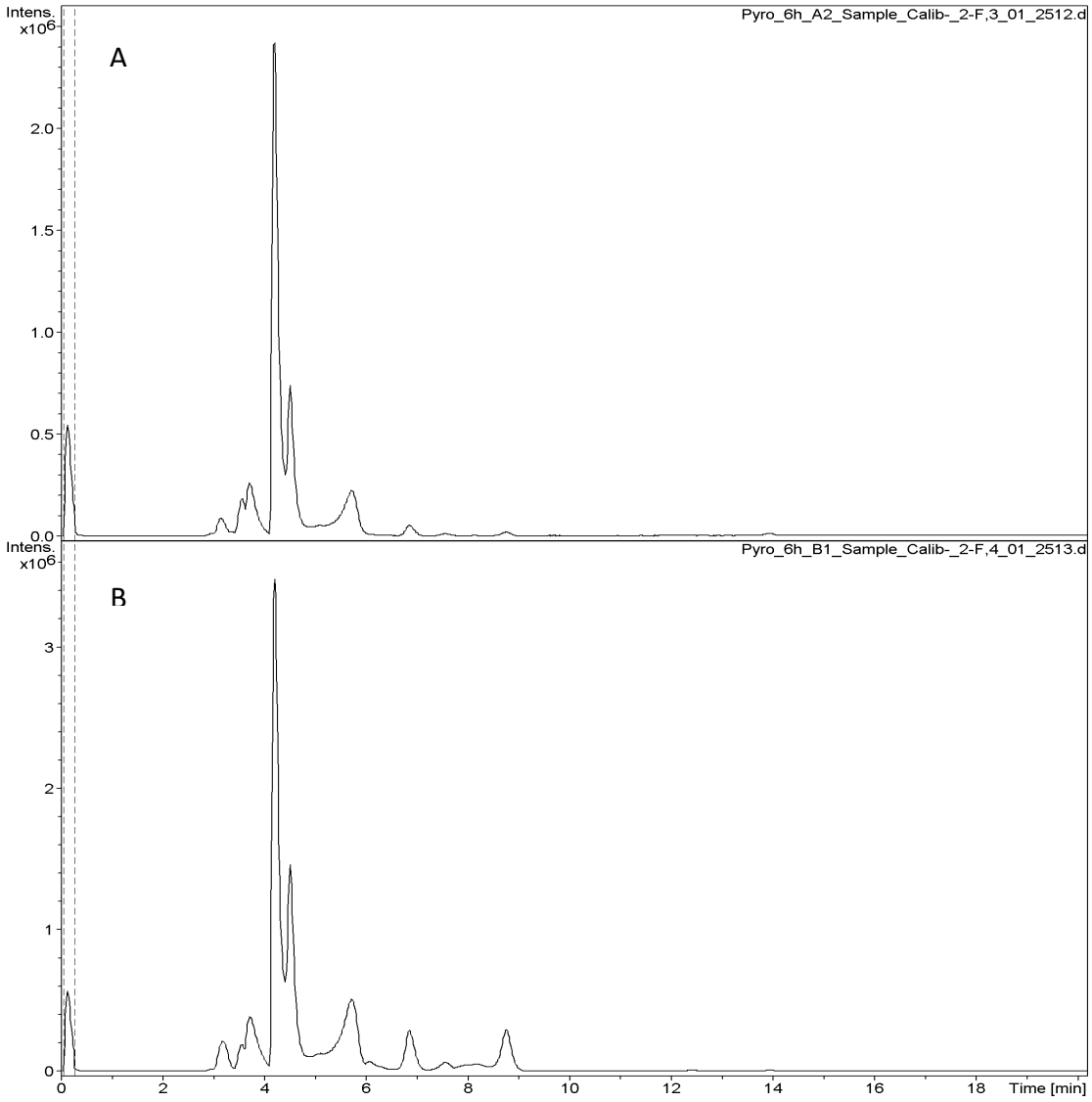

Figure 1: HPLC profile obtained with MS detector of pyrogallol-sulfate at 6 hours in apical (A) and basolateral (B) samples.

These results were observed in other samples, as for catechol-sulfate, 2-*O*-methyl pyrogallol sulfate and at other timepoints (6h and 24h). The possible explanation for not detecting the previous identified metabolites could be samples instability, metabolites could be degraded or the column used in the current analysis not be the more appropriate for these compounds.

At this stage it was decided to analise a new set of samples to take advantage of the remaining time. These samples have been obtained after *in vitro* digestion of leaves of *Arbutus unedo* and previous results indicate a great potential for the metabolites, still unknown, in models of Parkinson disease. These samples were dissolved in two different solvents, MeOH and water/acetonitrile (80:20) and the same results were observed in both solvents (Fig 2).

Methodologies: The column used in analyses was BEH C18 Waters (2.1x50 mm, 1.7µm). The mobile phases were solution A (0.1% (v/v) aqueous formic acid) and solution B (0.1% (v/v)

formic acid in acetonitrile/water (50:50, v/v). The flow rate was 450 µL min-1, and the gradient was 0 min; 97% A, 0- 3 min; 85% A, 3-7 min; 75% A, 7-10 min; 50% A, 10-13 min; 50% A, 13-14 min 0% A, 14-16 min 0% A, 16-17 min 97% A, 17-20 min 97% A). The PDA detector range was 200-600 nm. Mass spectrometer mass range was m/z 80-2000 with alternative full scan MS scans in negative mode

The identification of the compounds was made by comparison with retention time and exact mass obtained in previous analyzes in Orbitrap. In this case, it was possible to confirm several compounds in samples as epicatechin, quercetin derivatives, myricetin derivatives, kaempferol derivatives and others.

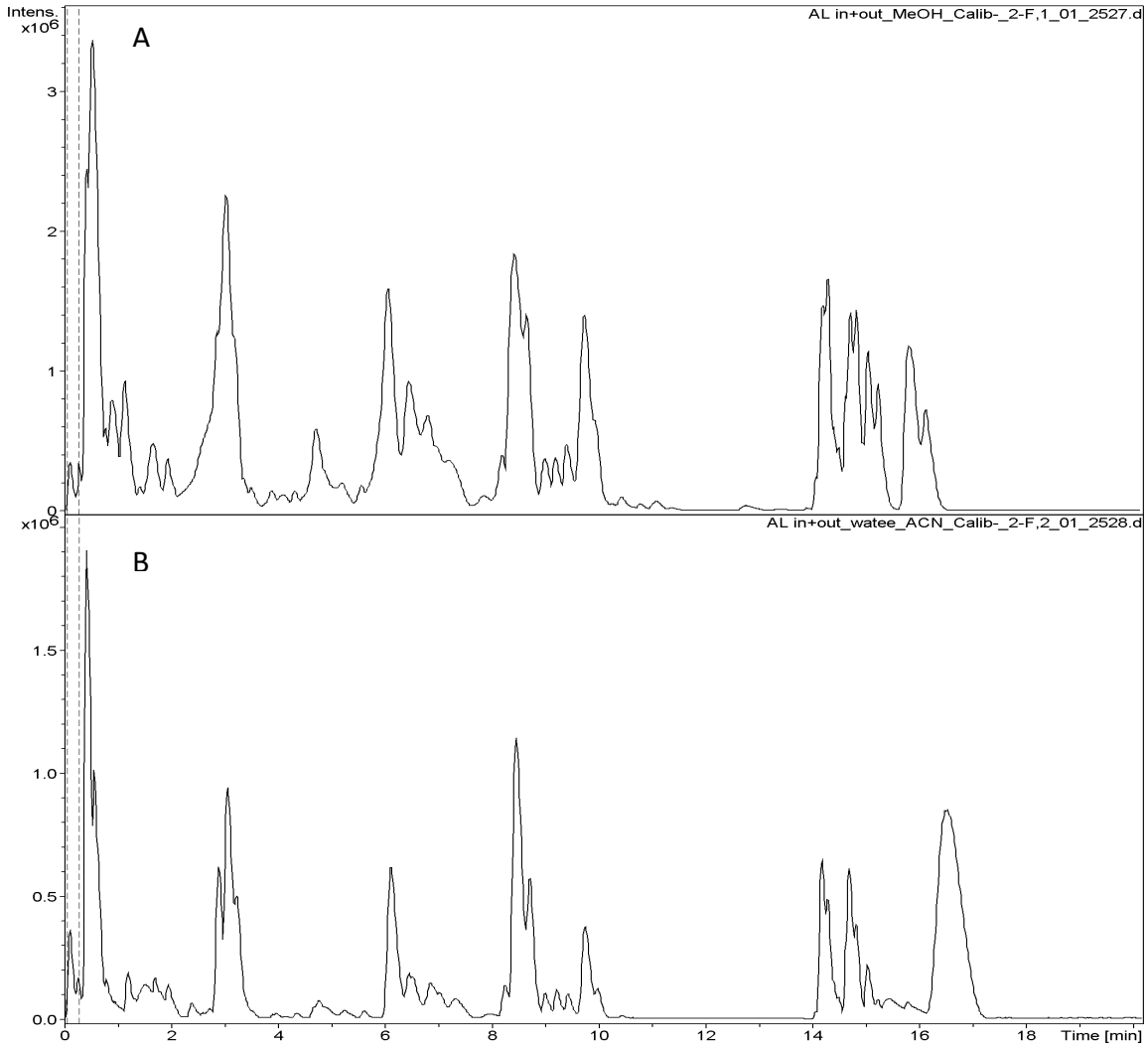

Figure 2 HPLC profile obtained with MS detector of *Arbutus unedo* samples in MeOH (A) and ACN (B).

Table 1 Identification of metabolites presents in *Arbutus unedo* samples.

Compound	Retention time (min)	*m/z* experimental	Molecular Formula $[M-H]^-$
Epicatechin	2.8	289.0717	$C_{15}H_{13}O_6$
Quercetin hexoside	6.8	463.0880	$C_{21}H_{19}O_{12}$

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Quercetin pentoside	8.2	433.0764	$C_{20}H_{17}O_{11}$
Myricetin-galloyl-hexoside	5.2	631.0936	$C_{28}H_{23}O_{17}$
Myricetin-3-rhamnoside	6.7	463.0880	$C_{21}H_{19}O_{12}$
Kaempferol hexoside	8.4	447.0922	$C_{21}H_{19}O_{11}$
Kaempferol pentoside	9.0-9.2	417.0812	$C_{20}H_{17}O_{10}$
Kaempferol rhamnoside	9.2	431.0969	$C_{21}H_{19}O_{10}$
Strictinin	4.7	633.0740	$C_{27}H_{21}O_{18}$
Digalloyl shikimic acid	4.1	477.0674	$C_{21}H_{17}O_{13}$
Unknown a	3.1	423.0933	$C_{19}H_{19}O_{11}$
Unknown b	3.9	431.1920	$C_{20}H_{31}O_{10}$
	6.0	431.1189	$C_{18}H_{23}O_{12}$
Unknown c*	4.0	405.1396	$C_{17}H_{25}O_{11}$
Unknown d	6.0	391.1033	$C_{19}H_{19}O_{9}$

However, the sample had four unknown peaks that it was necessary to determine the structure of the compounds. In a first approach, the compound *unknown a* is arbutin gallagic acid ester but this study needs to be finalized with further analyses.

This Short Term Scientific Mission allowed to create a new collaboration between iBET and CEBAS-CSIC, which can be very useful in future work and becoming an advantage for COST Action. I learned a new technique (LC-MS-SPE-NMR) and its potentialities for my future research work. I also had the opportunity to discuss and exchange new scientific ideas with the group members of the host laboratory. This is very important as my research is based on a multidisciplinary approach, combining diverse research fields, such as organic chemistry, analytical chemistry and biological and plant sciences.

The work developed in the two laboratories will be published in international scientific papers.

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