A Foodomics Approach: CE-MS for Comparative Metabolomics of Colon Cancer Cells Treated with Dietary Polyphenols

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i. Summary/Abstract

The potential of capillary electrophoresis-mass spectrometry (CE-MS) for Metabolomics is demonstrated through the analysis of metabolites from human HT29 colon cancer cells treated and non-treated with dietary polyphenols. Prior to CE-MS analysis, four different metabolite purification strategies are investigated. Namely, the results obtained after methanol deproteinization, ultrafiltration and two solid phase extraction (SPE) methods using C18 and polymer-based cartridges are described. These generic methods can have broad applications to analyse metabolites in a large variety of matrices and fields, including the new Foodomics area.

ii. Key Words

Foodomics, Metabolomics, colon cancer, dietary polyphenols, CE, CE-MS

1. Introduction

One of the main goals in modern food science and nutrition is to improve our limited understanding of the roles of nutritional compounds at molecular level. Dietary polyphenols are currently receiving considerable interest for their presumed role in the prevention of various degenerative diseases such as cancer and cardiovascular diseases (1). However, the molecular mechanism underneath the biological activity of these compounds and their cellular targets are still unknown. Foodomics has emerged as a new discipline that studies the food and nutrition domains through the application of advanced omics technologies in order to improve consumers well-being, health and confidence (2). The advent of “omics” techniques - interpreted broadly as a suite of high-throughput technologies for the generation, processing, and application of scientific information about the composition and functions of genome, proteome and metabolome- has created unprecedented opportunities for increasing our understanding of how nutrients modulate gene and protein expression and ultimately influence cellular metabolism. In order to gain detailed insight on the molecular mechanisms governing the metabolic pathways Metabolomics is a must in the new Foodomic field. Metabolome can be defined as the full set of endogenous or exogenous low molecular weight metabolic entities of approximately <1000 Da (metabolites), and the small pathway motifs that are present in a biological system (3). Considering the great diversity and heterogeneity of metabolites, simultaneous purification and analysis of all metabolites from a biological sample is also a challenge. Sample preparation for Metabolomics will depend on the type of sample being analyzed, the subsequent method of analysis and the metabolomic approach (targeted or non-targeted). As a result, at present no single analytical methodology or platform is available to detect, quantify and identify all metabolites in a certain sample, although it is now generally accepted that MS-based techniques (together with NMR) are crucial for Metabolomics.

However, due to the great complexity of metabolomes in biological samples an analytical separation technique is usually coupled to MS to avoid signal suppression and subsequent loss of metabolic information. In this regard, capillary electrophoresis (CE) is particularly suited for the rapid separation of ionic and polar compounds with very high resolution using extremely small reagents and sample volumes. The coupling CE-MS is described in this work for metabolite fingerprinting (4). TOF mass analyzer is used in the CE-MS coupling due to its high scan speed and high mass resolution and accuracy, what perfectly fits with the narrow peaks provided by CE. In summary, four different sample preparation procedures for cytosolic metabolite extraction from human HT29 colon cancer cells are described in this work, together with a complete CE-MS approach for the study of the effect of dietary polyphenols on colorectal cancer cells metabolome.

2. Materials

2.1. Reagents and samples

1. Distilled water from Milli-Q system (Millipore, Bedford, MA, USA)
2. MS grade water from Scharlau (Barcelona, Spain)
3. Methanol from Sigma Aldrich (St. Louis, MO).
4. Hydrochloric Acid (37%) from Sigma Aldrich.
5. Formic Acid (98-100%) from Riedel-de Haën (Seelze, Germany).
6. MS grade 2-propanol from Riedel-de Haën.
7. NaOH from Sigma Aldrich.
8. Sodium formate from Sigma Aldrich.
9. Trizma[®] base from Sigma Aldrich.
10. EDTA from Sigma Aldrich.
11. NaCl from Sigma Aldrich.

12. Phosphate buffered saline (PBS) solution containing 138 mM sodium chloride, 2.7 mM potassium chloride and 10 mM sodium hydrogen phosphate, at pH 7.4, from Sigma Aldrich.
13. Protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin, from Sigma Aldrich.
14. Amino acids (arginine, lysine, histidine, γ -aminobutyric acid (GABA), valine, serine, leucine, threonine, glutamine, proline, aspartic acid and tyrosine) from Sigma Aldrich.
15. Tyramine from Sigma Aldrich.
16. DL-methionine sulfone from Sigma Aldrich.
17. 1,4-piperazinediethanesulfonic acid (PIPES) from Sigma Aldrich.
18. Human HT29 colon cancer cells from Universidad de Miguel Hernandez (Elche, Spain).
19. Dulbecco's Modified Eagle Medium (DMEM) from Sigma Aldrich.

2.2. Solutions for sample treatment, metabolite extraction and CE-ESI-TOF MS method

1. Homogenization buffer solution (10 mM Tris-HCl, 5 mM EDTA, 120 mM NaCl, at pH 7.4) for sample treatment. To prepare 100 mL: Dissolve 1.21 g of Trizma® base, 1.87 g of EDTA and 3.51 g of NaCl in 95 mL of Milli-Q water and adjust the pH to 7.4 by adding HCl. Then reach a final volume of 100 mL by adding Milli-Q water using a volumetric flask.
2. Sodium formate (5 mM) for MS calibration. To prepare 100 mL: Mix 5 mL of 1 M NaOH with 99.5 mL of H₂O:2-propanol (50:50 v/v) with 0.2 % formic acid. To prepare 100 mL of 1 M NaOH: Dissolve 4 g of sodium hydroxide in 95 mL of Milli-Q water. Then reach a final volume of 100 mL using a volumetric flask.
3. Amino acids solution to corroborate metabolite identification. Amino acids are dissolved in Milli-Q water at the following concentrations: Arginine (0.58 mg/mL), lysine (0.49 mg/mL), histidine (0.52 mg/mL), γ -aminobutyric acid (GABA) (0.34 mg/mL), valine (0.39 mg/mL),

serine (0.35 mg/mL), leucine (0.44 mg/mL) , threonine (0.40 mg/mL), glutamine (0.49 mg/mL), proline (0.38 mg/mL), aspartic acid (0.44 mg/mL), tyrosine (0.09 mg/mL).

4. Formic acid 1 M at pH 1.8. To prepare 100 mL: Mix 3.77 mL of formic acid with 95 mL of Milli-Q water in a volumetric flask then reach a final volume of 100 mL.

5. Isopropanol:water (50:50 v/v). Mix 20 mL of MS grade 2-propanol with 20 mL of MS grade water.

6. Water-methanol (95:5, v/v). Mix 95 mL of Milli-Q water with 5 mL of methanol.

2.3. Instrumentation for sample preparation extraction procedures

1. Polytron[®] homogenizer from Capitol Scientific (Austin, TX)
2. Evolute[™] ABN columns from Biotage (Cardiff, Wales, UK).
3. Isolute C18 Endcapped cartridges from Biotage.
4. Amicon Ultra 3 kDa centrifugal device from Millipore (Bedford, MA, USA).
5. Savant SpeedVac SC 200 from Thermo Fisher Scientific (Waltham, MA, USA).

2.4. Instrumentation for CE-ESI-TOF MS analysis

1. High-performance capillary electrophoresis (CE) system model P/ACE 5500 from Beckman (Fullerton, CA, USA) controlled by a PC equipped with System Gold software from Beckman.
2. Syringe pump from Cole Palmer (74900-00-05) (Vernon Hills, IL, USA).
3. Uncoated fused-silica capillaries (50 µm id and 90 cm total length) from Composite Metal Services (Worcester, England)
4. Orthogonal electrospray interface model G1607A from Agilent Technologies (Palo Alto, CA, USA).
5. TOF MS instrument (micrOTOF) from Bruker Daltonics (Bremen, Germany) controlled by a PC equipped with micrOTOF control software from Bruker Daltonics.

2.5. Software for data processing and analyzing

1. DataAnalysis 3.3 software from Bruker Daltonics.
2. Free available databases used for tentative identification (5-9):

Database HMDB (<http://www.hmdb.ca/>)

Metlin (<http://metlin.scripps.edu/>)

KEGG compound (<http://www.genome.jp/kegg/compound/>)

KEGG Pathway (<http://www.genome.jp/kegg/pathway.html>)

PubChem (<http://pubchem.ncbi.nlm.nih.gov/>)

3. Methods

3.1. Sample preparation

1. Grow human HT29 cells in DMEM supplemented with 5% heat-inactivated fetal calf serum, 2 mM of L-glutamine, 50 U/ml of penicillin G and 50 µg/ml of streptomycin, at 37 °C in humidified atmosphere and 5 % CO₂. Treat the cell culture with polyphenols for 24 h.
2. Wash human HT29 colon cancer cells with PBS solution and then centrifuge them to form the pellets.
3. Suspend the pellets with homogenization buffer and protease inhibitor cocktail.
4. Disrupt the cells with a polytron homogenizer and centrifuge for 14 min at 14000 g and 4°C.
5. Discard the pellet (nuclear fraction) and take the supernatant (cytosolic fraction).
6. Centrifuge the supernatant for 1h at 100000 g and 4°C.
7. Store the supernatant (cytosolic fraction) at -80° C until metabolite purification procedure is carried out. This temperature stops enzyme activity and allow samples to be stored safely without continuing metabolic activity.

3.2. Metabolite extraction procedures

3.2.1. Solid-phase extraction (SPE)

1. Use EvoluteTM ABN columns and Isolute C18 Endcapped cartridges for SPE. The conditions of elution are identical for both solvents.
2. Pass 1 mL of methanol followed by 1 mL of water through the cartridges.
3. Add 350 μ L of Milli-Q water to 150 μ L of cytosolic fraction obtained from the cell culture.
4. Load this solution onto the column and wash with 1 mL of water-methanol (95:5, v/v).
5. Use 500 μ L of methanol to elute the sample.
6. Aliquot eluted sample in 100 μ L volume.
7. Vacuum dry the aliquots and store the dried extracts at -80°C.
8. Prior to CE-MS analysis, dissolve dried extracts in 20 μ L of MS grade water.

3.2.2. Protein precipitation

1. Add 350 μ L of methanol to 150 μ L of cytosolic fraction obtained from the cell culture.
2. Incubate the solution at -20°C during 2h.
3. Centrifuge the suspension for 5 min at 20000 *g* and 4°C.
4. Discard the pellet and collect the supernatant fraction.
5. Aliquot supernatant fraction in 100 μ L volume.
6. Vacuum dry the aliquots and store the dried extracts at -80°C.
7. Prior to CE-MS analysis, dissolve dried extracts in 20 μ L of MS grade water.

3.2.3. Ultracentrifugation

1. Add 350 μ L of Milli-Q water to 150 μ L of cytosolic fraction obtained from the cell culture.
2. Using the Amicon Ultra 3 kDa centrifugal device centrifuge the solution for 70 min at 14000 *g* and 20°C.

3. Collect with a pipette the fraction with molecular weight lower than 3 kDa and aliquot in 100 μ L volume.
5. Vacuum dry the aliquots and store the dried extracts at -80°C .
6. Prior to CE-MS analysis, dissolve dried extracts in 20 μ L of MS grade water.

3.3. CE-ESI-TOF MS metabolic analysis of human HT29 colon cancer cells

1. Condition all new capillaries by flushing with 0.1 M NaOH for 20 min, followed by water for 20 min and background electrolyte (BGE) for 5 min (*see Note 1*). Use next BGE composition for CE separation: 1 M formic acid (pH 1.8) (*see Note 2*).
2. After each run, condition the capillary with Milli-Q water for 4 min, followed by BGE for 4 min (*see Note 3*).
3. Set the interface between CE and TOF MS via a sheath-flow configuration, in which electrical contact at the electrospray needle tip is established via a flow of conductive sheath liquid with next composition: isopropanol-water (50:50 v/v) (*see Note 4*), and delivered at a flow rate of 0.24 $\mu\text{L}/\text{min}$ by a syringe pump. Use next nebulizer and drying gas conditions: 0.4 bar N_2 and 4 L/min N_2 , respectively, and maintain the ESI chamber at 200°C (*see Note 5*).
4. Operate the micrOTOF to acquire spectra in the m/z range of 50-600 every 90 ms. Make external and internal calibration of the TOF MS instrument (*see Note 6*) by introducing 5 mM sodium formate solution through the separation capillary.
5. Make sample injection at the anodic end using N_2 pressure of 0.5 psi (34.5 mbar) for 80 s (*see Note 7*).
6. Use +25 kV as running voltage at a constant temperature of 25°C to carry out electrophoretic separation by using 1 M formic acid as BGE.
7. Analyze each sample by triplicate by CE-MS.

8. Depending on metabolite extraction method (*see* **Section 3.2**), four different base peak electropherograms (BPE) are obtained by CE-MS (**Fig. 1**).

Figure 1. CE-ESI-TOF MS base peak electropherograms (BPE) of the cytosolic fraction from HT-29 colon cancer cells after four different metabolite extraction procedures. (A) SPE extraction using Isolute C18 Endcapped cartridge. (B) SPE extraction using Evolute™ ABN cartridge. (C) Metabolite extraction using methanol deproteinization. (D) Metabolite extraction using ultrafiltration with an Amicon Ultra 3 kDa centrifugal device.

3.4. Metabolomic fingerprinting of human HT29 colon cancer cells treated with dietary polyphenols

Follow the CE-ESI-TOF MS metabolomic approach explained in **section 3.3** to obtain the metabolic fingerprinting from human HT29 colon cancer cells treated and non-treated with dietary polyphenols (**Fig. 2**).

Figure 2. CE-ESI-TOF MS extracted ion electropherograms (EIE) of the cytosolic fraction from human HT-29 colon cancer cells: control (left panel) and treated with dietary polyphenols (right panel). Metabolite purification is carried out through methanol deproteinization.

3.5. Data Analysis

1. Using the DataAnalysis 3.3 software, annotate the accurate m/z value, migration time and area from each peak.
2. Depending on established m/z differences, eliminate the redundant responses from same ion, such as isotopic peaks, fragments, adducts, dimers, etc. Remove the low reliability signals such as non peak-like shapes or spike noise.
3. As a result of using a high mass accuracy TOF MS instrument, Generate-MolecularFormula Editor in DataAnalysis 3.3 software is capable to provide a list of possible elemental formulas (with errors below 5-10 ppm).
4. Enlighten standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents, and compare the theoretical and the experimental isotopic pattern (Sigma-ValueTM) for increased confidence in the theoretical molecular formula.
5. Use some free available databases (*see section 2.5*) in order to work out tentative identification based on the obtained molecular formula.
6. Confirmation of amino acid identification is carried out by using commercial compounds (*see section 2.2*).
7. After analysis by CE-ESI-TOF MS of human HT29 colon cancer cells using different metabolite purification protocols, 71 and 80 different metabolites are detected in C18 and ABN extract, respectively. Similar extraction selectivity is obtained since 62 are common metabolites. On the other hand, 83 and 74 metabolites are detected in MeOH and ultrafiltrate extract, respectively. Similar extraction selectivity is obtained since 72 are common metabolites. Comparing ABN (80 metabolites) and methanol (83 metabolites) purification, different extraction selectivity is observed with only 48 common metabolites.

8. After data analysis of ABN and methanol extracts a total number of 115 different metabolites with molecular masses from 90.0 to 777.2 Da are observed. 44 metabolites are tentatively identified and classified in several groups. Most abundant compounds are amino acids (histidine, arginine, lysine, GABA, valine, serine, leucine (or isoleucine), threonine, glutamine, proline, tyrosine and aspartic acid) and polypeptides, although other compounds such as lipoic acid derivatives, steroid derivatives, carbohydrates derivatives, amino alcohol, amino acid phosphates, nucleosides, quaternary amines, pterins, acyl phosphates and nucleotides, are also present. Among all tentatively identified compounds, 20 of them are related to the following metabolic pathways: glycan biosynthesis and metabolism, organismal systems: excretory system, neuroactive ligand-receptor interaction, carbohydrate metabolism, lipid metabolism, nucleotide metabolism, energy metabolism, metabolism of cofactors and vitamins, environmental information processing, genetic information processing and amino acid metabolism.

9. After CE-ESI-TOF MS analysis of metabolite extracts (obtained through methanol deproteinization) human HT29 colon cancer cells treated and non-treated with dietary polyphenols were compared. 90 different compounds are detected with this method in less than 20 min (see **Fig. 2**). Significant differences are found for ions: 130.1602 m/z, 132.0772 m/z, 212.1167 m/z, 240.1487 m/z, 271.0557 m/z, 118.0870 m/z, 150.0586 m/z, 166.0861 m/z, 182.0802 m/z and 307.0840 m/z. Among them, creatine, valine, tyrosine and glutathione disulfide were identified.

Notes:

1. Since the presence of non-volatile components decreases the MS sensitivity, increases the background noise and, under extreme conditions can clog the system, it must be used volatile sheath liquids and separation BGEs while working with MS.

2. A low pH should be used for the analysis in order to avoid analyte adsorption onto the inner capillary wall and confer positive charge to the analytes to improve their ESI ionization yield. Under these conditions, most amino acids, amines, nucleosides, small peptides, and in general, basic compounds, present overall positive charge and they migrate before EOF. Moreover, using these electrophoretic conditions allows to detect some acidic compounds (bearing some negative charge in their structure) being carried to the MS by the residual EOF.
3. In order to eliminate adsorbed compounds from the capillary wall, after each analysis, the capillary is rinsed.
4. Even some different mixtures of ACN, methanol, and isopropanol with water could be used, in order to obtain the best response on sensitivity and signal stability of metabolites, 50:50 (v/v) isopropanol-water mixture is recommended as sheath liquid for the electrospray interface.
5. Even the spray is stable between 2 and 6 $\mu\text{L}/\text{min}$, it is recommended to use 4 $\mu\text{L}/\text{min}$ as sheath liquid flow rate to reach the best S/N value during CE-ESI-TOF MS analysis of metabolites. Nebulizer pressure (0.2-0.7 bar) has also some effect on MS sensitivity, a nebulizer pressure of 0.4 bar is advised to obtain the best results.
6. External and internal calibration of the TOF MS instrument should be performed prior to each analysis. Masses for calibration of the TOF MS instrument are next: 90.9766, 158.9641, 226.9515, 294.9389, 362.9263, 430.9138, 498.9012 and 566.8886 m/z.
7. Since different salt concentration of the purified samples may change the amount of injected sample, use pressure injection instead of electrokinetic injection. By applying 0.5 psi pressure for 80 s, a sample plug of 2.7 cm (3% of the capillary length) is injected.

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