

Dilute-and-shoot-based direct nano-electrospray ionization tandem mass spectrometry as screening methodology for multivitamins in dietary supplement and human urine



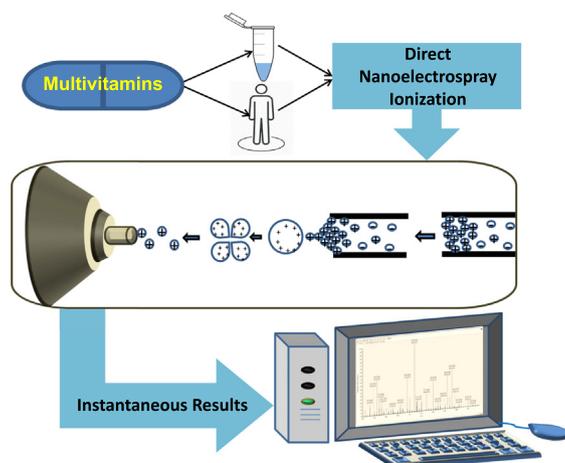
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ABSTRACT

Introduction: In recent years, analytical screening methods for simultaneous detection of multivitamins have gained substantial attention to ensure quality and public confidence in dietary supplements. Even so, few analytical methods have been proposed for simultaneous analysis of multivitamin constituents due to the large divergence in chemical characteristics.

Objectives: In the present study, the objective was to develop a simple and rapid direct nano-electrospray ionization-tandem mass spectrometry (DI-nano-ESI-MS/MS) method for targeted detection of water soluble vitamins, fat soluble vitamins, amino acids, royal jelly, ginkgo biloba, and ginseng in a dietary supplement. The applicability of dilute-and-shoot-based DI-nano-ESI-MS/MS to analyze the same tested compounds and their related metabolites in clinical samples was also examined.

Methods: Intact urine mixed with the ionization solvent was loaded (4- μ L aliquot) into a nanospray (NS) capillary of 1- μ m tip diameter. The NS capillary was then fitted into an off-line ion source at a distance of

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Royal jelly
Human urine

5 mm from MS aperture. The sample was directly injected by applying a voltage of 1.1 kV, producing a numerous of m/z peaks for analysis in mere minutes.

Results: The DI-nano-ESI-MS/MS method successfully identified almost all dietary supplement components, as well as a plethora of component-related metabolites in clinical samples. In addition, a new merit of the proposed method for the detection of index marker and chemical contaminants as well as sub-species identification was investigated for further quality evaluation of the dietary supplement.

Conclusions: The previous findings illustrated that DI-nano-ESI-MS/MS approach can emerge as a powerful, high throughput, and promising analytical tool for screening and accurate detection of various pharmaceuticals and ingredient in dietary supplements as well as biological fluids.

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Introduction

As analytical methods advance, the significance of vitamins in even minute pathways within the human body has become more and more apparent. Most of these vitamins are not synthesized in the body, making it crucial for one to consume a balanced diet. Even so, the nature of the average consumer diet's nutritional value is deteriorating, causing a need for multivitamin dietary supplements. The exponential increase in the sales of dietary supplements in the last two decades has raised a cause for concern on their quality control. The current market's multivitamins cover a large array of essential vitamins, amino acids and minerals. Amino acids such as arginine, lysine, L-carnitine, and L-glutamine are the basis of all bodily processes and make up a large portion of the body's tissues, especially muscle [1,2]. Water soluble B vitamins and ascorbic acid (vitamin C) and their circulating metabolites are involved in energy metabolism and play roles in muscles, skin, eyes, hair, liver maintenance and immune system [3–5]. Fat soluble vitamins are stored in fat tissue, and are essential to one's vision, immune system, bone maintenance and blood regulation [6–9].

Other natural products have now become quite popular in the consumer market, and include ginkgo biloba, ginseng and royal Jelly. Ginkgo biloba has been documented as a neuroprotective and been used in cases of Alzheimer's, and has beneficial effects on cardiovascular diseases, cancer, memory loss, and psychiatric disorders [10]. Ginseng has been promoted for its adaptogenic properties and used to treat atherosclerosis, bleeding disorders, and has hypoglycemic, hypotensive, and hypolipidemic properties [11]. Similarly, royal jelly is also known for its hypoglycemic, hypotensive, and hypolipidemic properties along with a plethora of other pharmacological activities including antioxidant, hepatoprotective, anti-inflammatory, anti-tumor, anti-allergic, and anti-aging [12].

Classical HPLC methods with ultraviolet [13], photodiode array [14] and fluorescence [15] detection systems are still used routinely to determine vitamins in different types of samples. However, all of these methods are not confirmatory approaches, because they cannot provide direct proof of the analytes structures. Undoubtedly, the introduction of liquid chromatography-mass spectrometry (LC-MS) [16] and gas chromatography-mass spectrometry (GC-MS) [17] was a major step towards analysis of multivitamins in different matrices. The obvious advantage of MS is that it is a global quantum detector of any compound. Liquid chromatography-mass spectrometry (LC-MS) methods have become ubiquitous in screening laboratories due to its higher sensitivity, specificity, and ability to provide structural information. Even so, when applying LC-MS in biological matrices, there are still some disadvantages in the efficiency and stability of ionization due to the complexity and diversity of matrices, and in effect, only a few methods have been applied to determine multivitamins simultaneously in bio-fluid samples [18,19]. It is apparent that the most common LC-MS methods for monitoring analytes in biological

matrices are time-consuming because of sample-pretreatment procedures that are usually based on liquid-liquid extraction [20] or solid-phase extraction [21] for the cleaning and/or concentration of the analytes. Thus, there is an urgent need to develop a rapid and direct MS screening technique to improve detection efficiency while maintaining adequate sensitivity.

Recently, nano-ESI systems have become popular tools in the sample ionization process of MS analysis. Even so, few studies have taken advantage of this system in vitamin analysis, specifically using nano-chips and desorption electrospray ionization [22,23]. The nanospray (NS) capillary, manufactured by Humanix (Hiroshima, Japan), has been developed as a new direct coupling technique with MS in order to identify various intra-cellular bio-active compounds [24–29]. With continuous investigations, these NS capillaries can be emerged as a powerful and promising analytical tool for screening and accurate detection of various pharmaceutical compounds. To the best of our knowledge, the present study is the first method that utilizes NS capillaries as a replacement for LC-MS interface system in the development of direct injection nano-electrospray ionization-tandem mass spectrometry (DI-nano-ESI-MS/MS) technique for multivitamins analysis. Thus, it was important to investigate the applicability of the DI-nano-ESI-MS/MS technique for targeted detection of fat soluble vitamins, water soluble vitamins, amino acids and other botanical ingredients simultaneously within a dietary supplement. The generalizability of the DI-nano-ESI-MS/MS approach to detect the same tested analytes and their related metabolites in human urine was also conducted using a dilute-and-shoot procedure without sample pretreatment and chromatographic separation.

Experimental

Instruments

MS analysis was achieved with a high resolution Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with an off-line NS ion source. The NS ion source was located on a manual XYZ podium that was placed in the front of MS entrance. An aliquot of 4 μL sample solution was loaded into NS capillaries of 1 μm tip pore size (Humanix, Hiroshima, Japan) using pipette tips (Eppendorf, GELoader tips). Once the NS capillary was installed on the ion source, then the XYZ phase was used to shift the capillary to the optimal position. A video camera has been placed between the tip of the capillary and the MS entry, allowing the analyst to monitor and calculate the distance between the two. MS analysis was carried out in both positive and negative modes under ambient conditions with an ion spray voltage of ± 1.1 kV and capillary temperature of 250 $^{\circ}\text{C}$. The scan method was divided into the following m/z ranges: 100–1000, 50–100, 100–130, 130–160, 160–200, 200–250, 250–300, 300–350, 350–400, 400–500, 500–600, 600–700, 700–800, 800–900, 900–1000, 1000–1500; and each segment was scanned for 20 s. Selected m/z values were fragmented at collision induced dissociation (CID) of 25.

Chemicals and reagents

HPLC grade methanol was used (Tokyo Chemical Industry Co., LTD, Tokyo, Japan). Formic acid (Sigma-Aldrich, Munich, Germany) was of reagent grade ($\geq 95\%$). Ultrapure water (Organo, Puric- ω , Tokyo, Japan) was daily prepared and used in all experiments.

Pharmaceutical formulations

Kerovit[®] soft gelatin capsules (Amoun Pharmaceutical Company S.A.E.) were purchased from a local pharmacy, and contain 10 mg of arginine ethyl ester, 10 mg of lysine, 10 mg of L-carnitine, 10 mg of L-glutamine, 10 mg of choline bitartrate, 20 mg of inositol, 5 mg of co-enzyme Q 10, 50 mg of lecithin, 5 mg of phosphatidylserine, 30 mg of eicosapentaenoic acid, 20 mg of docosahexaenoic acid, 5 mg of royal jelly, 25 mg of ginseng, 25 mg of bee pollen, 25 mg of wheat germ oil, 45 mg of soyphosphatides, 15 mg of safflower oil, 20 mg of ginkgo biloba extract, 3000 IU of vitamin A (palmitate), 200 IU of vitamin D, 200 IU of vitamin E, 80 μg of vitamin K, 60 mg of vitamin C, 3 mg of vitamin B1 (as mononitrate), 20 mg of vitamin B2, 15 mg of vitamin B3, 600 μg of B6, 100 μg of vitamin B9, 60 μg of vitamin B12, 10 mg of vitamin B7, 30 mg of vitamin B5, 9 mg of calcium, 13.5 mg of iron, 150 μg of phosphorus, 5 mg of iodine, 7.5 mg of magnesium, 2 mg of zinc, 200 μg of copper, 2 mg of selenium, 120 μg of nickel, and 130 μg of vanadium.

Application

Dosage form analysis

A capsule of Kerovit[®] was emptied and washed with 5 mL of 60% methanol and centrifuged for one minute. An aliquot of 100 μL of the supernatant was mixed with 100 μL of ionization solvent (100 μL of 80% methanol containing 3 μL of formic acid). A minute volume of 4 μL was loaded into the rear of the NS tip, centrifuged for a few seconds, and fixed onto the off-line ion source for ionization and MS detection.

Urine sampling

A healthy 26 year old female volunteer was administered a single dose of Kerovit[®] at 8P.M. with a glass of water after emptying the bladder and was instructed not to eat anything until the next morning. The next day, fresh morning urine samples were collected at 8 A.M. in clear polypropylene centrifuge tubes and immediately stored under -80°C . An aliquot of 100 μL of ionization solvent was added to 100 μL of intact urine sample after filtering through a 0.22 μm Millex[®] GV filter unit. A minute of 4 μL of the sample solution was then loaded into the rear of the NS tip, and analyzed as mentioned under the dosage form section.

Data analysis

Data analyses were performed using the XCalibur software (Thermo Fisher Scientific, San Jose, USA). Detected peaks were identified by the exact mass value of ± 5 part per million (ppm) tolerance, or difference between calculated and found m/z values calculated as the difference in calculated and found m/z values divided by the calculated m/z value times one million, and annotated by KEGG database (<http://www.kegg.jp/kegg/>) and HMDB database (<http://www.hmdb.ca/>) using an in-house excel macro program for automatic identification. Only database hits larger than a signal to noise ratio (S/N ratio) of 3 were used in identification, both signals of which were automatically calculated by the software. For parent compounds, peaks of interest were analyzed by MS/MS for molecular identification. The obtained fragment peaks were identified by referring to MetFrag web tool ([\[msbi.ipb-halle.de/MetFrag/\]\(http://msbi.ipb-halle.de/MetFrag/\)\) and MassBank database \(<http://www.massbank.jp/>\). Metabolite identification was done by parent peak, isotope, and/or adduct \$m/z\$ hits by the excel macro and confirmed by the presence in documented literature.](http://</p>
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Results and discussion

Method development and optimization

Compared to LC-MS methods, DI-nano-ESI-MS/MS parameters are less in number, but even so, any change could affect the end results. Along with the absence of LC, the proposed method is done under ambient conditions, thereby eliminating the conditions such as flow rate, turbo gas conditions, cone voltage, declustering potential, entrance potential, curtain gas collision, and gas temperature. In contrast, the major parameters that required optimization in the current study included ionization solvent composition, capillary voltage, MS inlet temperature, and capillary distance to MS inlet. Regarding the constituents of ionization solvent, 80% methanol containing 3 μL of formic acid was found to be convenient for the ionization of nearly all tested analytes. Capillary voltage was tested within a range of ± 0.8 –1.3 kV and the total ion counts (TIC) of runs at each measured voltage were compared. A potential of ± 1.1 kV gave the highest TIC in positive and negative modes, and therefore was chosen as the optimum capillary voltage. Concerning MS inlet temperature, a range of 200–400 $^\circ\text{C}$ was tested in increments of 50 $^\circ\text{C}$. No noticeable difference was observed, except that noise levels increased after 300 $^\circ\text{C}$. Therefore, 250 $^\circ\text{C}$ was the chosen MS inlet temperature. Finally, the distance between NS tip and MS inlet needed to be optimized. This was carried out through the use of a manual XYZ podium to adjust the NS tip position, and a live video camera in order to measure the distance on the spot. It was observed that a distance of 5 mm resulted in the highest TIC in both positive and negative ionization modes.

Dosage form targeted analysis:

Targeted detection of amino acids

Detection of amino acids has been described by ESI-MS and showed the possibility of their positive as well as their negative ionization [30]. Thus, DI-nano-ESI-MS seems a useful tool for analysis of amino acids in multivitamins dosage form. All four amino acids, arginine, lysine, L-carnitine and L-glutamine, present in the dosage form were detected and confirmed using the proposed method. The full scan spectrum in positive ionization mode yielded protonated molecular ion peaks $[\text{M}+\text{H}]^+$ at m/z 203.1503, 147.1129, 162.1125, and 147.0765 for arginine ethyl ester, lysine, L-carnitine and L-glutamine, respectively. Fig. 1 depicts all four amino acids in a full mass spectrum, while (S1, Supplementary material) depicts the MS/MS spectrum of each one. L-glutamine (S1-A) loses a water molecule, producing its major fragment at m/z 130.1010, and loses a carbonyl group and an additional water molecule to produce a minor fragment at m/z 84.1007. In contrast, lysine (S1-B) initially loses an amino group to produce its minor fragment at m/z 130.1002, and then, similar to L-glutamine, loses a carbonyl group and water molecule to produce its major fragment at m/z 84.1014. L-carnitine (S1-C) also loses a water molecule upon fragmentation (m/z 144.6995), and also fragments to form 3-carboxy-2-hydroxyprop-1-ylum at m/z 103.1017 and N,N-trimethylmethanaminium at m/z 60.0402. As for arginine (S1-D), its presence in the ethyl ester form causes it to have a unique fragmentation pattern. The parent compound initially is fragmented by losing its guanidine entity, producing its major fragment at m/z 144.2430, and the free protonated guanidine is shown at m/z

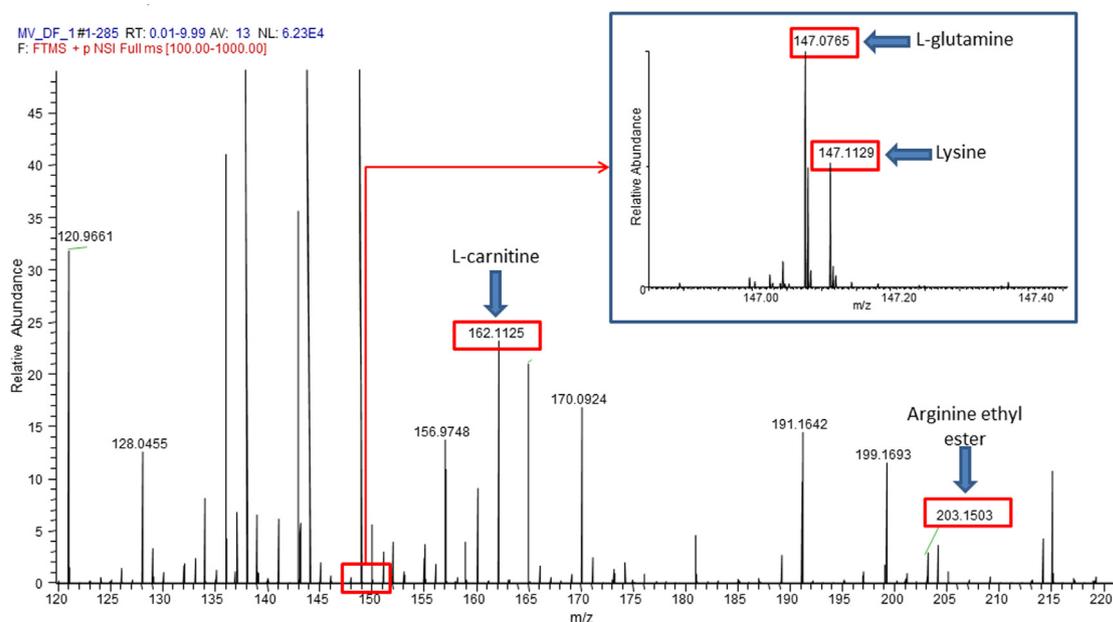


Fig. 1. Full mass spectrum depicting all four amino acids.

60.0201. The fragment of m/z 144.2430 is further fragmented at the amino-carbonyl bond to form but-3-en-1-iminium at m/z 70.2086. All these fragmentation patterns were similarly produced by Piraud et al [30] for method reliability.

Targeted detection of fat soluble vitamins

DI-nano-ESI-MS/MS was carried out for detection and confirmation of fat soluble vitamins and the results are illustrated in **S2-A & B**. In positive ionization mode, vitamin A, of palmitate ester, was observed as a protonated peak at m/z 525.4667. Under CID, it also produced a minor fragment at m/z 507.4085 due to dehydration and a major fragment at m/z 269.2520 corresponding to $[M+H-palmitate]^+$. Under negative mode of ionization, vitamin E had a more stable deprotonated parent peak $[M-H]^-$ at m/z 429.3732, and its fragmentation followed the classical Retro-Diels-Alder fragmentation pattern [31] in which the bonds between O-1 and C-2 and C-3 and C-4 are broken, producing the major fragment at m/z 163.0711.

Targeted detection of water soluble vitamins

All water soluble vitamins including thiamine (B1), riboflavin (B2), nicotinamide (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9), cyanocobalamin (B12), and ascorbic acid were successfully detected using the proposed method, whether in positive or negative ionization mode, and most produced several ionized species. The MS/MS product ion experiments were performed in the mode which produced the parent peak of highest S/N ratio for the confirmation of each vitamin. All MS measurements were within 3 ppm of theoretical exact mass, and MS/MS spectra were matched with available literatures. Briefly, choline was detected in the positive ion mode as $[M]^+$ at m/z 104.1075 and produced three main fragments peaks (in order of intensity) at m/z 60.0779, 58.0103, and 59.0011, which match the results of Xiong et al [32]. Nicotinamide was found in the positive mode as $[M+H]^+$ at m/z 123.0552 and upon fragmentation produced four main fragments at m/z 80.0509, 78.0211, 96.0296, and 105.0401, and these results agree with Wang et al [33]. Pyridoxal and Pyridoxine were matched to fragmentation patterns cited by Leporati et al [34] and Chen Z, Chen B, and Yao [35]. Pyridoxal ($[M+H]^+$ at m/z 168.0654) produced major fragments at m/z 150.2207 and 122.1247, while pyridoxine ($[M+H]^+$ at m/z

170.0812) produced three fragments at m/z 152.3321, 134.1091, and 124.2088, all under positive ion mode. Ascorbate was found with high intensity under negative ion mode $[M-H]^-$ at m/z 175.0248, and produced three major fragments at m/z 115.0703, 89.0074, and 69.0398; these results agree with Szultka et al [36]. Pantothenate was detected in the positive ion mode as $[M+H]^+$ at m/z 220.1182 and produced major fragments at m/z 202.1331, 184.1399, and 90.0507, all which match the results of Wolf et al [37]. Biotin was detected in the negative ion mode as $[M-H]^-$ at m/z 243.0810 and produced only two main fragments at m/z 200.0691 and 166.0871, which agree with the pattern produced by Kumar et al [38]. Thiamin was found in the positive ion mode as $[M]^+$ at m/z 265.1125 and produced two main fragments at m/z 122.2111 and 144.1423, matching the results of Steckel and Schlosser [39]. Riboflavin was also found in the positive ion mode as $[M+H]^+$ at m/z 377.1452 and two main fragments at m/z 243.2189 and 359.0626 were produced, agreeing with both Leporati et al [34] and Redeuil et al [40]. Folic acid was detected in positive ion mode as $[M+H]^+$ at m/z 442.1469 and produced four main fragments at m/z 295.0501, 176.4431, 424.0269, and 313.0592. This pattern agrees with those produced by Nelson, Sharpless, and Sander [41] and Holčapek and Jandera [42]. Cyanocobalamin was detected in positive ion mode as $[M+2H]^{2+}$ at m/z 678.2909 and upon fragmentation produced three fragments at 359.2410, 341.1647, and 636.0225, which agree with the findings of Chas-saigne and Łobiński [43]. These results are summarized in Table 1, and MS/MS spectra are depicted in **S3-S12**.

Royal jelly

Targeted detection of royal jelly index marker and other related compounds. Royal jelly contains a plethora of components that aid in maintaining human health, but the presence of *trans*-10-hydroxy-2-decenoic acid (10-HDA), the major fatty acid component, is considered to be the marker that serves as an index for quality of royal jelly products [44,45]. Therefore, it is essential to detect 10-HDA in the dosage form for confirmation of royal jelly presence. The proposed DI-nano-ESI-MS/MS method detected and identified 10-HDA, with a base peak $[M-H]^-$ at m/z 185.1183, and upon fragmentation, produced two main daughter peaks at m/z 167.1116 and 141.0881 corresponding to water and carbon dioxide loss, respectively, along with a peak at m/z 71.0106 of

Table 1
Identification of water soluble vitamins by MS/MS.

Vitamin	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	Fragments	Other species found *	Ref
Choline	104.1075	+	[M] ⁺	104.1075	104.1072	2.88	60.0779(1 0 0) 58.0103(27) 59.0011(13)	None	[32]
Nicotinamide (B3)	122.0480	+	[M+H] ⁺	123.0552	123.0553	-0.82	80.0509(1 0 0) 78.0211(32) 96.0296(24) 105.0401(9)	[M-H] & [M+HCOO]	[33]
Pyridoxal	167.0582	+	[M+H] ⁺	168.0654	168.0655	-0.60	150.2207(1 0 0) 122.1247(6)	[M-H] & [M+HCOO]	[34,35]
Pyridoxine (B6)	169.0740	+	[M+H] ⁺	170.0812	170.0813	-0.59	152.3221(1 0 0) 134.1091(27) 124.2088(9)	[M-H] & [M+HCOO]	[34]
Ascorbate	176.0320	—	[M-H] ⁻	175.0248	175.0249	-0.57	115.0703(1 0 0) 89.0074(17) 69.0398(13)	[M-H(¹³ C)], [M+Cl], [M+ ³⁷ Cl], [M+H] ⁺ , [M+K] ⁺	[36]
Pantothenate (B5)	219.1110	+	[M+H] ⁺	220.1182	220.1181	0.46	202.1331(1 0 0) 184.1399(23) 90.0507(63)	[M+H] ⁻ , [M-H(¹³ C)], [M+Na] ⁺	[37]
Biotin (B7)	244.0882	—	[M-H] ⁻	243.0810	243.0810	0.00	243.0810(1 0 0) 200.0691(64) 166.0817(42)	[M+Cl] & [M+ ³⁷ Cl], [M+H] ⁺ , [M+Na] ⁺	[38]
Thiamin (B1)	265.1120	+	[M] ⁺	265.1120	265.1125	-1.89	122.2111(1 0 0) 144.1423(58)	[M-H] ⁻ , [M-H(¹³ C)]	[39]
Riboflavin (B2)	376.1380	+	[M+H] ⁺	377.1452	377.1455	-0.80	243.2189(1 0 0) 359.0626(14)	[M+Na] ⁺ , [M+K] ⁺ , [M-H], [M-H(¹³ C)], [M+Cl], [M+ ³⁷ Cl]	[34,40]
Folic acid (B9)	441.1397	+	[M+H] ⁺	442.1469	442.1467	0.45	295.0501(1 0 0) 176.4431(54) 424.0269(13) 313.0592(7)	[M-H] & [M+HCOO]	[41,42]
Cyanocobalamin	1354.5674	+	[M+2H] ²⁺	678.2909	678.2887	1.62	359.2410(1 0 0) 341.1647(26), 636.0225(14)	None	[43]

* Detection at positive or negative ionization mode.

the remaining 2-propenoic acid fragment. This MS/MS spectrum is depicted in **S13**, and effectively matches the tandem mass profile produced by Caparica-Santos and Marcucci [46]. Other fatty acids documented to be in royal jelly [46] were also detected in negative mode ionization, including the deprotonated ions of 10-hydroxydecanoic acid (10-HDAA), at m/z 187.1339, along with the minor fatty acids known as sebacic acid at m/z 201.1134, 12-hydroxydodecanoic acid at m/z 215.1657, traumatic acid at m/z 227.1289, and dodecanedioic acid at m/z 229.1445. These results illustrate that, DI-nano-ESI-MS/MS can serve as a new approach to detect and confirm quality markers for authenticity identification and quality evaluation of highly complex chemical compositions.

Identification of royal jelly chemical contaminants. Because royal jelly is grown, collected, and harvested, it contains not only a vast variety of active constituents, but also has the possibility of containing contaminants, either purposely or not. Therefore, constituent such as this raw material may carry an extra burden in regards to analysis in the sense that dietary supplements screening trials are required to ensure the presence of declared ingredients and to exclude the presence of undeclared or unauthorized ingredients [47]. Thus, it was important to evaluate the promising performance of the DI-nano-ESI-MS/MS method to clarify its ability for detection of chemical contaminants. Besides compounds found in the tested dietary supplement, the proposed DI-nano-ESI-MS/MS method could also detect several quinolone antibiotics, which have been documented as common royal jelly contaminants due to their application inside the beehives [48]. This investigation could gain high importance for the evaluation of royal jelly authenticity as a component of the dietary supplement. All detected antibiotics benefited the formation of higher intensity and stability of Cl⁻ adducts beside the formation of formate adduct in case of ciprofloxacin, as summarized in **Table 2**. The mass spectrum obtained using negative ionization mode showed that balofloxacin, moxifloxacin and levofloxacin/ofloxacin could be detected as deprotonated molecular ions [M-H]⁻ at m/z 388.1693, 400.1682 and 360.1382, respectively. Under the same condition, norfloxacin,

ciprofloxacin and levofloxacin/ofloxacin also showed isotopic molecular ion peaks [M+³⁷Cl] at m/z 356.0989, 368.099 and 398.1099, respectively.

Ginseng detection and subspecies identification

While over 150 ginsenoside derivatives (ginseng saponins) have been identified, Rb1, Rb2, Rc, Rd, Re, and Rg1 are the major ginsenosides, which constitute 80–90% of total saponins [49]. The present DI-nano-ESI-MS/MS method could identify four ginsenosides, within the tested dietary supplement, including ginsenoside Rb1, ginsenoside Re, ginsenoside Rg1 and notoginsenoside R1. The full mass spectrum in negative ion mode yielded deprotonated molecules [M-H]⁻ at m/z 799.4833 (ginsenoside Rg1), 931.5256 (notoginsenoside R1), 945.541 (ginsenoside Re) and 1107.5957 (ginsenoside Rb1). Under the same conditions, ginsenoside Re is distributed over two isotopic molecular peaks at m/z 946.5442 [M-H(¹³C)] and 947.5471 [M-H(¹³C)₂], while ginsenoside Rb1 showed one isotopic molecular peak [M-H(¹³C)] at m/z 1108.6018. These results are summarized in **Table 3**.

It is well known that ginsenosides are found exclusively in different *Panax* (*P*) species, mainly *P. quinquefolius* (American ginseng), *P. ginseng* (Chinese ginseng), and *P. notoginseng* (Notoginseng) [50]. Thus, many analytical methods have been reported to differentiate between these subspecies. Through these methods, several chemical differences have been established. Notably, pseudoginsenoside F11 is present in American ginseng while ginsenoside Rf and notoginsenoside R1 are present in both of Chinese ginseng and Notoginseng. In addition to these findings, the ratio of ginsenoside Rg₁/Rb₁ has been widely accepted as an indication of species. A ratio value of less than 0.4 corresponds to American ginseng, while a value higher than 0.4 is an indication of Chinese ginseng and Notoginseng [51]. Accurate identification of herbal ingredients is essential for developing safe and effective dietary supplements that meet consumer expectations. Thus, the developed DI-nano-ESI-MS/MS method was applied for such purpose and the results showed that the tested dietary supplement contains Chinese ginseng and Notoginseng subspecies, and the Rg₁/Rb₁ ratio was calculated to be 0.68.

Table 2
Identification of quinolone antibiotics in the dietary supplement.

Metabolite	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	S/N Ratio
Norfloxacin	319.1332	—	$[M+Cl]^-$	354.1020	354.1019	0.28	42.09
			$[M+^{37}Cl]^-$	356.0990	356.0989	0.28	12.1
Levofloxacin/Ofloxacin	361.1437	—	$[M-H]^-$	360.1365	360.1382	-4.72	17.02
			$[M+Cl]^-$	396.1126	396.1123	0.76	39.57
Ciprofloxacin	331.1330	—	$[M+^{37}Cl]^-$	398.1096	398.1099	-0.75	10.18
			$[M+Cl]^-$	366.1021	366.1018	0.82	25.24
			$[M+^{37}Cl]^-$	368.0991	368.0990	0.27	5.98
Balofloxacin	389.1751	—	$[M+HCOO]^-$	376.1309	376.1330	-5.58	13.83
			$[M-H]^-$	388.1679	388.1693	-3.61	33.96
			$[M+Cl]^-$	424.1439	424.1436	0.71	3.89
Moxifloxacin	401.1751	—	$[M-H]^-$	400.1679	400.1682	-0.75	15.36
			$[M+Cl]^-$	438.1415	438.1409	1.37	21.48

Table 3
Identification of ginseng components in the dietary supplement.

Metabolite	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	S/N Ratio
Ginsenoside Rg1	800.4922	—	$[M-H]^-$	799.4842	799.4833	1.13	46.29
			$[M+HCOO]^-$	845.4902	845.4908	-0.71	30.66
Notoginsenoside R1	932.5345	—	$[M-H]^-$	931.5265	931.5256	0.97	116.2
			Ginsenoside Re	946.5501	946.5501	—	—
Ginsenoside Rb1	1108.6029	—	$[M-H]^-$	945.5421	945.5410	1.16	191.32
			$[M-H(^{13}C)]^-$	946.5453	946.5442	1.16	92.68
			$[M-H(^{13}C)_2]^-$	947.5485	947.5471	1.48	26.4
			$[M-H]^-$	1107.5957	1107.5957	0.00	67.89
			$[M-H(^{13}C)]^-$	1108.5989	1108.6001	-1.08	11.56

Targeted detection of ginkgo biloba

Ginkgo biloba is among the top 10 botanical dietary supplements. For centuries it has been used as a traditional Chinese medicine to treat various diseases such as asthma, alcoholism and bladder infections. Currently, ginkgo biloba leaf powders and extracts are used to treat circulatory disorders [52]. Among the many components in ginkgo biloba, flavonoids, terpenic lactones (ginkgolides) and terpenoid (bilobalide) are the most relevant.

During negative ion electrospray MS, the developed method could detect several $[M-H]^-$ peaks of ginkgo flavenoids, such as kaempferol, quercetin, isorhamnetin and rutin at m/z 285.0406, 301.0349, 315.0501, and 609.146, respectively. Conversely, in the positive ion mode, the major terpenic lactones, ginkgolide A, ginkgolide B, and ginkgolide C produced protonated peaks $[M+H]^+$ at m/z 409.1488, 425.1442, and 441.1387, respectively. In negative mode ionization, these three compounds did not produce deprotonated peaks, but instead produced peaks corresponding to the formate adducts $[H + HCOO]^-$ at m/z 453.14, 469.1331, and 485.1295, respectively. Also, bilobalide was detected in negative mode of ionization as parent deprotonated peak and formate adduct at m/z 325.0928, 371.0984, respectively. The observation in the use of both positive and negative ionization modes, along with the identification and use of adducts in both modes, has been previously established and used extensively in ginkgo biloba analysis [53].

Urine analysis

Merits of dilute-and-shoot-based DI-nano-ESI-MS/MS

It is well known that, screening of multivitamins simultaneously in human urine is difficult and represents several analytical problems because of the diversity of their chemical structures and properties. Thus, it is a challenge to identify them in a single run method. Also, vitamins are excreted in urine at relatively low concentrations and thus they required sensitive and selective methods for their analysis. Deleting or reducing sample cleanup and fast analysis can shorten the run time as well as cost saving in reagents and consumables. As a result, a procedure called "dilute-and-shoot" can be employed for a large scale screening.

However, untreated matrix can lead to noticeable matrix effects and diminish instrument performance. DI-nano-ESI-MS/MS method can make this strategy more attractive because NS mechanism creates a more stable signal and allows high sensitivity. So, the more sensitive the system, the more likely the sample can be diluted, and thereby reducing the matrix's effects and thus sample pretreatment procedures could be eliminated. The unique shape of NS capillary and its minute tip diameter (1 μ L) allow for optimum ESI and small droplet size, resulting in a higher degree of sample ionization. The minute volume approach can allow for the introduction of less matrix components into the MS system, consequently extending the useful life of the equipment, unlike conventional LC methods. In addition, the use of disposable NS capillary minimizes the possibility of contamination and carry-over effect. Moreover, while ion adducts are more pronounced in NS ionization, the present work uses them as further confirmation of detected metabolites, as previous studies have proved their usefulness, even to the extent of quantification [54,55]. The merit of the method also lies within the singularity of the NS tip. Conventional LC-MS methods require each run to consume a new volume of sample for a single purpose. In contrast, only one NS tip carrying a very minute volume is enough to be considered as a sample reservoir for extract total investigative parameters. An aliquot of 4 μ L sample volume is enough for over 15 min of scan time due to the nL/min flow rate, allowing for simultaneous full scan investigation, detection, identification, and MS/MS confirmation of multiple compounds, all done under the same conditions without the probability of carry over or sample-to-sample contamination. Therefore, a single NS capillary gives the power of multi-injection purposes with the flexibility of various MS related variables (m/z range, MS level, resolution) in real time within the same run.

Selectivity

Selectivity was assessed by screening six drug-free urine samples and monitoring the absence of interferences arising from matrix components with the detection of targeted compounds. Safety in the identification was also performed by comparing the results of drug-free urine samples with that of human clinical urine

samples after oral administration of the multivitamins capsule. The study showed that direct detection of multivitamins and their related metabolites as well as other botanical ingredients in human urine is feasible by DI-nano-ESI-MS/MS method despite the presence of interfering components arising from urine matrix.

Screening of amino acids and their metabolites

DI-nano-ESI-MS seems a useful tool to improve detection limits of amino acids and minimizes ion suppression effects with a complex matrix such as urine due to the lower injected volume. MS/MS was an efficient tool in the present study for the confirmation of the tested amino acids in dietary supplement. Consequently, it was easy to apply the developed DI-nano-ESI-MS method successfully for screening of amino acids and their predictable metabolites in clinical urine samples by dilute-and shoot procedure after administration of a single oral dose of the tested dietary supplement. Of the four amino acids present in the tested capsule, two were detected during analysis of urine as deprotonated molecular ion peaks [M-H] at m/z 145.0983 and 145.0619 for lysine and L-glutamine, respectively. As for L-carnitine, it was not detected, which was expected due to its numerous roles in metabolic pathways and extensive renal reabsorption [56]. Although arginine itself was not detected, numerous metabolites were found along with their respective Cl⁻ adducts, [M+Cl]. All metabolites detected and their corresponding peaks are summarized in Table 4. Mass spectrum of arginine metabolites in negative ion mode yielded deprotonated molecules [M-H] at m/z 187.1201 (homoarginine), 289.1154 (L-argininosuccinate) and showed characteristic chloride adducts [M+Cl] at m/z 225.0769 (homoarginine), 225.0769 (hydroxylarginine) and 325.0925 (L-argininosuccinate), all of which have been previously reported [57]. Lysine metabolites under the same condition yielded three deprotonated molecules [M-H] at m/z 131.0351, 187.1088 and 203.1038 for glutarate, N-acetyl-L-lysine and N₆-acetyl-N₆-hydroxy-L-lysine, respectively, and which have been described by Piraud et al [30]. The signals of L-glutamine metabolites are distributed over three isotopes [M-H(¹³C)] at m/z 147.0493, 189.0598 and 264.1073 and three deprotonated molecules [M-H] at m/z 146.0461, 188.0565 and 263.1039 for L-glutamate, N-acetyl-L-glutamate and phenylacetylglutamine, respectively. L-glutamine also showed a characteristic formate adduct pattern [M+HCOO] at m/z 309.1092, all of which have been reported also by Piraud et al [30].

Screening of fat soluble vitamins and their metabolites

Of the fat soluble vitamins present in the multivitamin formula, only vitamin A was detected in urine. This observation may be due to two main reasons: (1) the low initial concentration of the other labeled vitamins, and (2) the low percentage of their excretion into urine due to lipophilic properties. Unlike the other fat-soluble vitamins, vitamin A has acidic properties and therefore a relatively high percentage is excreted into urine. Also, peaks corresponding to metabolites of palmitate were observed, which we concur to directly correspond to retinol as a result of the original pharmaceutical formula containing retinol as a palmitate-ester form. Fig. 2 compares MS spectra of blank urine (A) and samples taken at 8 A.M. (B) showing that the palmitate metabolites were absent or negligible prior to multivitamin ingestion, further confirming the applicability of DI-nano-ESI-MS/MS method for the detection of vitamins after dosing. Table 5 lists all retinol-related compounds and their mass data.

The signals of vitamin A metabolites were distributed over multiple entities of isotopes, adducts and deprotonated molecules in the mass spectrum. In negative ion mode, human urine sample yielded deprotonated molecules with measured accurate mass of [M-H] at m/z 269.2123 (16-oxo-palmitate) and 269.2487 (methyl palmitate). Their [M-H(¹³C)] isotopic peaks were also detected at

m/z 270.2156 and 270.252, respectively. Retinoyl glucuronide produced a [M-H] peak at m/z 475.2335 and showed a formate adduct [M+HCOO] at m/z 521.2394. In the positive ion mode, 4-oxo-13-cis-retinoate showed characteristic [M+K]⁺ and [M+Ca]⁺ adducts at m/z 352.1440 and 353.1431, respectively. All these metabolites were detected and identified previously by Gundersen, and Blomhoff [58].

Screening of water soluble vitamins and their metabolites

All tested water soluble vitamins in the dietary supplement were detected in urine samples (at 8 A.M.) except for choline, folic acid, and cyanocobalamin. Previous studies on choline have reported that little to no amount is excreted in urine as the parent compound, but rather in its three main metabolites, trimethylamine oxide, dimethylglycine, and betaine [59]. In the case of folic acid, urine excretion occurs only if it present in excess in the human body, which, according to the Center for Disease Control, would not be less than 1 mg/day [60]. As for cyanocobalamin, we thought that it might be undetected due to its low initial dosage concentration. For the remaining vitamins, several m/z peaks were detected relating to different ion species/adducts of each vitamin. Metabolites of each vitamin were then identified in the full scan mode.

The full scan mass spectra obtained using negative ionization mode showed that the most abundant forms of deprotonated molecules [M-H] of vitamins B₃, B₆, B₅, B₁, B₂, B₇, and ascorbic acid were matched with the values detected in the dosage form at m/z 121.0168, 168.0667, 218.1035, 264.1049, 375.1298, 243.081, 179.0562 and 175.0249, respectively. Therefore, these eight deprotonated molecules have respectively been confirmed as the precursor ions of the corresponding vitamins. Under the same condition, vitamins B₅, B₂, and B₁ showed isotopic molecular ion peaks [M-H(¹³C)]. In positive ion mode, vitamins B₃, B₆, B₅, B₂ and ascorbic acid displayed protonated molecular ion peaks [M+H]⁺, while vitamins B₃ and B₆ showed formate adducts [M+HCOO] in negative ion mode. Vitamin B₂ and ascorbic acid showed [M+K]⁺ adducts as well as chloride isotopic molecular ion peaks [M+³⁷Cl]. Also, vitamin B₅ displayed sodium adduct [M+Na]⁺.

The observation of three distinct MS peaks both with measured accurate masses of [M+H]⁺ at m/z 76.0755, 104.0705 and 118.0862 within less than 3 ppm of the theoretical exact mass suggested the presence of choline metabolites, trimethylamine N-oxide, N, N-dimethylglycine and betaine, respectively, backed up by studies performed by Ocque, Stubbs, and Nolin [61]. Betaine also showed characteristic [M+Na]⁺ and [M+K]⁺ adducts at m/z 140.0680 and 156.0423, respectively.

In positive ion mode, seven distinct MS peaks of vitamin B₃ metabolites were detected as four protonated molecular ion peaks [M+H]⁺ at m/z of 137.0709 (methylnicotinamide), 138.0550 (methylnicotinate), 287.1003 (N-glucosylnicotinate) and 335.0647 (nicotinamide D-ribonucleotide), two adducts [M+Na]⁺ at 161.0327 (nicotinamide-N-oxide) and 326.1325 (nicotinamine) and one [M+K]⁺ adduct at 293.0613 (N-ribosylnicotinamide), all which have been previously reported [62,63]. In negative ion mode, the metabolites of vitamin B₆ were distributed over characteristic deprotonated molecular ion peaks [M-H] at m/z 164.0355 (pyridoxolactone), 166.0511 (pyridoxal), 182.0460 (pyridoxic acid) and 248.0327 (pyridoxine phosphate), all found and reported by Yagi et al [64]. Under the same conditions, vitamin B₆ also showed five adducts [M+HCOO] at m/z 210.041 (pyridoxolactone), 212.0565 (pyridoxal), 228.0515 (pyridoxic acid), 213.0881 (pyridoxamine) and 293.0546 (pyridoxamine phosphate).

In negative ion mode, mass spectrum revealed diagnostic deprotonated product ion at m/z 173.0093 and mono-isotopic molecular ion peak [M-H(¹³C)] at m/z 174.0126 for dehydroascor-

Table 4
Amino acid metabolites detected in urine.

Amino Acid	Metabolite	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	S/N Ratio
Arginine	Homoarginine	188.1273	—	[M-H] ⁻	187.1201	187.1200	0.53	31.56
			—	[M+Cl] ⁻	223.0967	223.0976	-4.03	4.99
	Hydroxyarginine	190.1066	—	[M+Cl] ⁻	225.0760	225.0769	-4.00	7.42
	L-Argininosuccinate	290.1226	—	[M-H] ⁻	289.1154	289.1154	0.00	13.94
			—	[M+Cl] ⁻	325.0920	325.0925	-1.54	6.31
Lysine	-	146.1055	—	[M-H] ⁻	145.0983	145.0983	0.00	26.05
	Glutarate	132.0423	—	[M-H] ⁻	131.0351	131.0352	-0.76	13.91
	N-Acetyl-L-lysine	188.1161	—	[M-H] ⁻	187.1089	187.1088	0.53	21.94
	N6-Acetyl-N6-hydroxy-L-lysine	204.1110	—	[M-H] ⁻	203.1038	203.1038	0.00	8.28
L-Glutamine	-	146.0691	—	[M-H] ⁻	145.0619	145.0621	-1.38	611.89
			—	[M-H(¹³ C)] ⁻	146.0652	146.0653	-0.68	26.86
	L-Glutamate	147.0532	—	[M-H] ⁻	146.0460	146.0461	-0.68	346.18
			—	[M-H(¹³ C)] ⁻	147.0493	147.0493	0.00	11.26
	N-acetyl-L-glutamate	189.0637	—	[M-H] ⁻	188.0565	188.0565	0.00	102.57
			—	[M-H(¹³ C)] ⁻	189.0598	189.0598	0.00	5.14
	Phenylacetylglutamine	264.1110	—	[M-H] ⁻	263.1038	263.1039	-0.38	4746.47
		—	[M-H(¹³ C)] ⁻	264.1071	264.1073	-0.76	640.46	
		—	[M+HCOO] ⁻	309.1090	309.1092	-0.65	643.41	

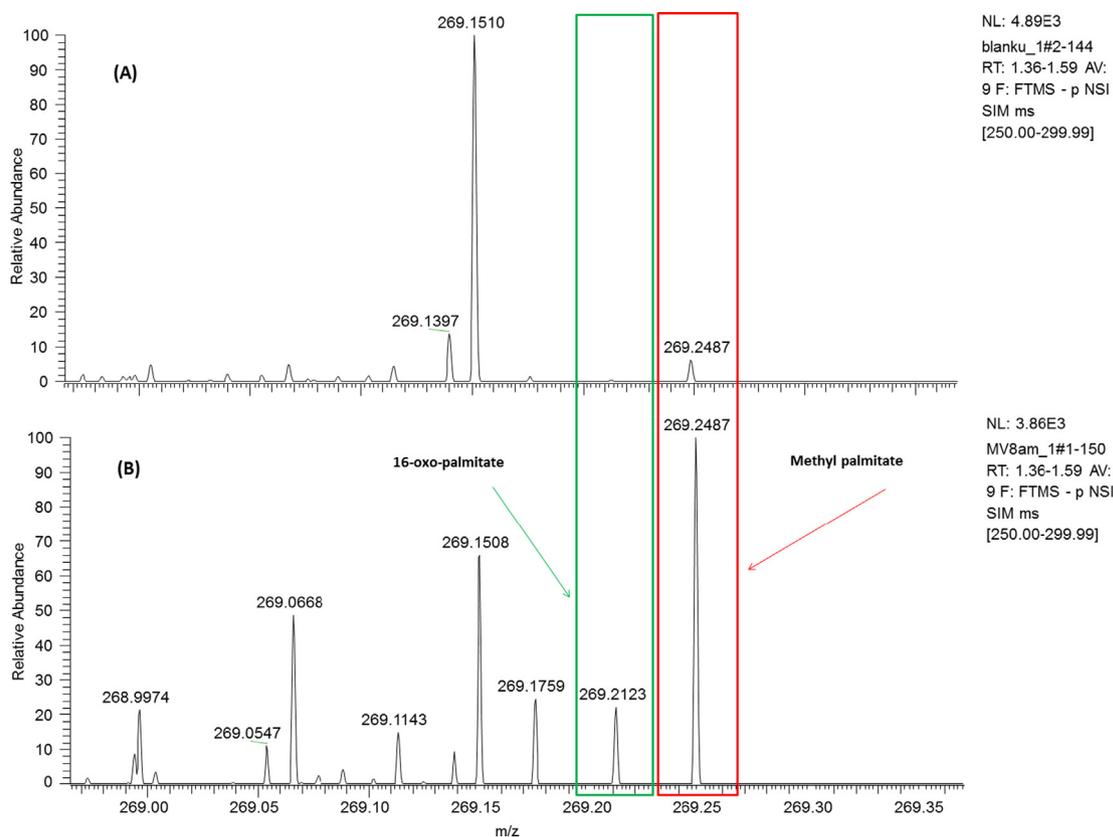


Fig. 2. Comparison of full mass spectrum of blank urine (A) and urine samples taken at 8 A.M. after vitamin A ingestion (B).

Table 5
Vitamin A metabolites detected in urine.

Metabolite	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	S/N Ratio
16-oxo-palmitate	270.2195	—	[M-H] ⁻	269.2123	269.2123	0.00	34.42
			[M-H(¹³ C)] ⁻	270.2156	270.2156	0.00	5.97
Methyl palmitate	270.2559	—	[M-H] ⁻	269.2487	269.2487	0.00	155.22
			[M-H(¹³ C)] ⁻	270.2520	270.2520	0.00	26.79
			[M+K] ⁺	352.1441	352.1440	0.28	35.33
4-oxo-13-cis-retinoate	313.1810	+	[M+Ca] ⁺	353.1436	353.1431	1.42	41.87
			[M-H] ⁻	475.2338	475.2335	0.63	57.43
Retinoyl glucuronide	476.2410	—	[M-H] ⁻	475.2338	475.2335	0.63	57.43
			[M+HCOO] ⁻	521.2390	521.2394	-0.77	5.25

bate, vitamin C metabolite. Detection of ascorbate-2-sulfate, another metabolite of vitamin C, at m/z of 256.9960 $[M+H]^+$, 254.9819 $[M-H]$, 255.9851 $[M-H(^{13}C)]$, 290.9582 $[M+Cl]$, and 292.9554 $[M+^{37}Cl]$ also confirms the presence of vitamin C. These metabolites have long been studied and established by the likes of Tolbert et al [65]. As for vitamin B5, three metabolites, dehydropantoate, pantoate, and pantolactone, were identified, in negative mode ionization. Dehydropantoate produced a main ion $[M-H]$ peak at m/z 145.0509 along with a monoisotopic peak and formate adduct at m/z 146.0541 and 191.0561, respectively. Similarly, pantoate also produced the same signal species at m/z 147.0665, 148.0698, 193.0718, respectively. In contrast, pantolactone only produced two peaks, both of adducts, at m/z 175.0612 and

165.0326, corresponding to formate $[M+HCOO]$ and chloride $[M+Cl]$ adducts, respectively. As for vitamin B7, two main metabolites, bisnorbiotin and biotinsulfone, were both detected as the parent $[M-H]$ signal and $[M-H(^{13}C)]$ isotopic peak at m/z 215.0496 and 216.0530 and m/z 275.0711 and 276.0748, respectively [66]. The vitamin B1 produced the choride adduct and chloride isotopic peak of thiamin monophosphate, $[M+Cl]$ and $[M+^{37}Cl]$, at m/z 380.0476 and 382.0456, respectively, the main urine metabolite described by Tallaksen et al [67]. Vitamin B2 metabolism produced reduced riboflavin, represented as $[M-H]$ and $[M-H(^{13}C)]$ peaks at m/z 377.1465 and 378.1497, and flavin mononucleotide, represented as its chloride adduct and respective isotope at m/z 491.0758 and 493.0722, respectively, both of which have been previously

Table 6

Water soluble vitamins metabolites detected in urine.

Vitamin	Metabolite	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	S/N Ratio	
Choline	Trimethylamine N-oxide	75.0684	+	$[M+H]^+$	76.07560	76.0755	1.31	114.57	
	N,N-dimethylglycine	103.0630	+	$[M+H]^+$	104.0702	104.0705	-2.88	9.34	
	Betaine	117.079	+	$[M+H]^+$	118.0862	118.0862	0.00	437.76	
			+	$[M+Na]^+$	140.0682	140.0680	1.43	148.55	
B3	Methylnicotinamide	136.0640	+	$[M+H]^+$	156.0421	156.0423	-1.28	12.67	
			+	$[M+H]^+$	137.0712	137.0709	2.19	495.6	
			+	$[M+H]^+$	138.0552	138.0550	1.45	2637.26	
			+	$[M+Na]^+$	161.0322	161.0327	-3.10	112.3	
	Nicotinamide- N- oxide	138.0430	+	$[M+K]^+$	177.0061	177.0063	-1.13	56.41	
			+	$[M+K]^+$	294.0611	294.0613	-0.68	14.04	
			+	$[M+H]^+$	287.1002	287.1003	-0.35	833.59	
			+	$[M+Na]^+$	309.0822	309.0825	-0.97	395.18	
	N-Ribosylnicotinamide	255.0980	+	$[M+K]^+$	325.0561	325.0564	-0.92	106.07	
			+	$[M+Na]^+$	326.1322	326.1325	-0.92	97.32	
	N-Glucosylnicotinate	286.0930	+	$[M+H]^+$	335.0642	335.0647	-1.49	27.71	
			+	$[M+Na]^+$	357.0462	357.0479	-4.76	7.88	
	B6	Pyridoxolactone	165.0430	—	$[M-H]^-$	164.0358	164.0355	1.83	35.22
				—	$[M-H(^{13}C)]^-$	165.0391	165.0389	1.21	4.31
—				$[M+HCOO]^-$	210.0412	210.0410	0.95	81.24	
—				$[M+HCOO]^-$	166.0508	166.0511	-1.81	25.94	
Pyridoxal		167.0580	—	$[M+HCOO]^-$	212.0562	212.0565	-1.41	15.18	
			+	$[M+H]^+$	168.0652	168.0654	-1.19	36.46	
			—	$[M-H]^-$	182.0458	182.0460	-1.10	192.89	
			—	$[M-H(^{13}C)]^-$	183.0491	183.0493	-1.09	13.76	
Pyridoxic acid		183.0530	—	$[M+HCOO]^-$	228.0512	228.0515	-1.32	36.52	
			—	$[M+HCOO]^-$	213.0882	213.0881	0.47	19.31	
			—	$[M+HCOO]^-$	293.0542	293.0546	-1.36	12.1	
			—	$[M-H]^-$	248.0328	248.0327	0.40	21.64	
Vit C		Dehydroascorbate	174.0164	—	$[M-H]^-$	173.0092	173.0093	-0.58	379.06
				—	$[M-H(^{13}C)]^-$	174.0125	174.0126	-0.57	22.21
	Ascorbate-2-sulfate	255.9890	+	$[M+H]^+$	256.9962	256.9960	0.78	28.23	
			—	$[M-H]^-$	254.9818	254.9819	-0.39	289.1	
			—	$[M-H(^{13}C)]^-$	255.9851	255.9851	0.00	17.25	
			—	$[M+Cl]^-$	290.9584	290.9582	0.69	21.82	
			—	$[M+^{37}Cl]^-$	292.9554	292.9554	0.00	6.91	
			—	$[M-H]^-$	145.0507	145.0509	-1.38	292.83	
			—	$[M-H(^{13}C)]^-$	146.0540	146.0541	-0.68	14.73	
			—	$[M+HCOO]^-$	191.0561	191.0561	0.00	168.69	
B5	Dehydropantoate	146.0579	—	$[M-H]^-$	147.0668	147.0665	2.04	959.74	
			—	$[M-H(^{13}C)]^-$	148.0701	148.0698	2.03	51.69	
			—	$[M+HCOO]^-$	193.0722	193.0718	2.07	3.48	
	Pantoate	148.0740	—	$[M+HCOO]^-$	175.0612	175.0612	0.00	61.12	
			—	$[M+Cl]^-$	165.0324	165.0326	-1.21	43.77	
			—	$[M+^{37}Cl]^-$	167.0294	167.0296	-1.20	5.09	
B7	Bisnorbiotin	216.0570	—	$[M-H]^-$	215.0498	215.0496	0.93	67.22	
			—	$[M-H(^{13}C)]^-$	216.0531	216.0530	0.46	7.86	
	Biotinsulfone	276.0780	—	$[M-H]^-$	275.0708	275.0711	-1.09	34.82	
			—	$[M-H(^{13}C)]^-$	276.0741	276.0748	-2.54	3.13	
B1	Thiamin monophosphate	345.0786	—	$[M+Cl]$	380.0480	380.0476	1.05	70.21	
			—	$[M+^{37}Cl]^-$	382.0450	382.0456	-1.57	25.79	
B2	Reduced riboflavin	378.1539	—	$[M-H]^-$	377.1467	377.1465	0.53	184.1	
			—	$[M-H(^{13}C)]^-$	378.1500	378.1497	0.79	28.99	
	Flavin mononucleotide	456.1050	—	$[M+Cl]^-$	491.0744	491.0758	-2.85	14.63	
			—	$[M+^{37}Cl]^-$	493.0714	493.0722	-1.62	3.32	
B9	5-Methyltetrahydrofolate	459.1866	—	$[M-H]^-$	458.1794	458.1790	0.87	4281.35	
			—	$[M-H(^{13}C)]^-$	459.1827	459.1833	-1.31	464.94	
			—	$[M-H(^{13}C)_2]^-$	460.1860	460.1868	-1.74	76.22	

discussed by Hustad et al [68]. Finally, observation of deprotonated molecular ion peak [M-H] at m/z 458.1790 suggested the presence of 5-methyltetrahydrofolate, a major metabolite of vitamin B9 [40]. Clear isotopic molecular ion peaks [M-H¹³C] and [M-H(¹³C)₂] at m/z 459.1833 and 460.1868, correlated to 5-methyltetrahydrofolate, have also been identified in negative ion mode, respectively. A summary of the water soluble vitamin metabolites are depicted in Table 6.

Screening of royal jelly metabolites

Several urine metabolites identified by Yamaga et al, [46] were detected by DI-nano-ESI-MS/MS method. Peaks corresponding to [M-H] for nonane-4,6-dione, 4-allylguaiacol, decenedioic acid, and 3-hydroxysebacic acid were detected at m/z 155.1078, 163.0765, 199.0976, and 217.1082, respectively. The present method also detected the parent acids 10-HDA and 10-HDAA. Both [M-H] (m/z 185.1187 and 187.1336, respectively) and [M+HCOO] (m/z 231.1244 and 233.1184, respectively) of these acids were detected in urine samples with adequate S/N ratio. MS/MS analysis was done to confirm presence of 10-HDA, which produced the same fragmentation patterns as that produced during dosage form

analysis, indicating that the developed method, while direct, is of high sensitivity.

Screening of ginseng and its metabolites

In negative ion mode, notoginsenoside R1 and ginsenoside Rb1 were detected in urine samples with similar molecular ion peaks [M-H] as found in the dosage form. The absence of ginsenoside Rg1 and ginsenoside Re is mainly due to their biotransformation by the intestinal microflora [69]. Previous researches to identify multiple ginseng metabolites in urine have been based on a multiple dosing regimen and in effect, cumulated concentrated samples, or have volunteers consume a large initial dose of high concentration [69,70]. In contrast, DI-nano-ESI-MS/MS method was performed after a single oral dose, and even so two main metabolites were detected. Under the same conditions, compound K (the most abundant metabolite derived from ginsenoside Rb1) and panaxytriol (a fatty alcohol of ginseng) were observed as deprotonated parent peaks at m/z 652.3509 and 277.1811, respectively. Under the same condition, compound K and panaxytriol showed their respective formate adducts at m/z 698.3561 and 323.1863, respectively (Table 7). These results are backed by

Table 7
Ginseng related compounds and metabolites detected in urine.

Compound	Exact mass	Ionization Mode	Ion species	Calc m/z	M/z found	Ppm diff	S/N Ratio
Notoginsenoside R1	932.5345	—	[M-H] ⁻	931.5265	931.5253	1.29	54.21
Ginsenoside Rb1	1108.6029	—	[M-H] ⁻	1107.5957	1107.5954	0.27	67.89
Compound K	653.3577	—	[M-H] ⁻	652.3505	652.3509	-0.61	45.87
			[M+HCOO] ⁻	698.3557	698.3561	-0.57	31.56
Panaxytriol	278.1882	—	[M-H] ⁻	277.1810	277.1811	-0.36	122.32
			[M+HCOO] ⁻	323.1862	323.1863	-0.31	89.77

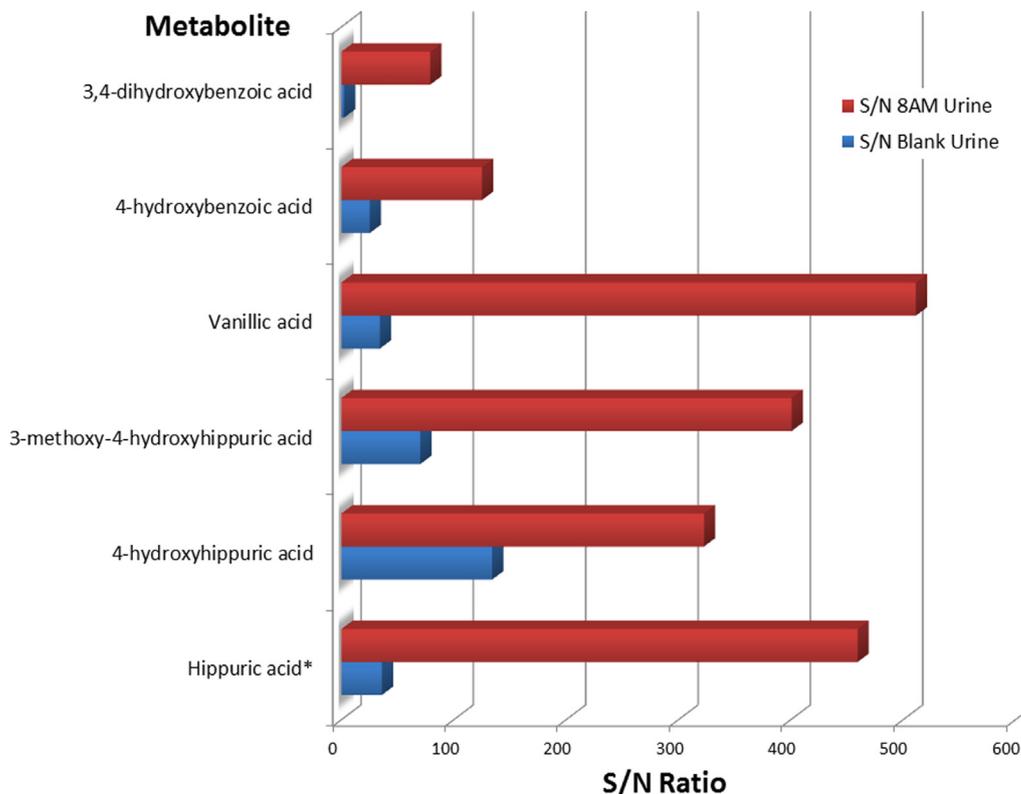


Fig. 3. Comparison of S/N ratio of selected ginkgo flavonol metabolites in blank urine and 8 A.M. urine samples. *S/N ratios of hippuric acid in blank and clinical urine samples were multiplied by a factor of 10^{-1} to fit graph scale.

several previous literature, including the use of the formate adducts for confirmation [71,72].

Screening of ginkgo biloba and its metabolites

Intact ginkgolides A and B and bilobalide were detected in urine samples during negative mode ionization as deprotonated peaks at m/z 407.1345, 423.1296, and 325.0928, respectively. In addition, both ginkgolide A and bilobalide formed stable formate adducts at m/z 453.1400 and 371.0984, respectively. These results agree with those of Dew et al. [73]. Rutinose, the rutin glycoside, was detected as its deprotonated molecule [M-H] at m/z 325.1139, and was confirmed by the characteristic chloride adduct [M+Cl] and isotopic pattern [M+H(³⁷Cl)] at m/z 361.0908 and 363.0878, respectively. Also, quercetin 3,3',7-trisulfate was detected and confirmed in urine sample through the identification of its parent deprotonated peak and ¹³C isotopic peak at m/z 540.9079 and 541.9114, respectively.

Flavenol metabolites studied by Pietta et al. [74] were identified with comparable results. Briefly, parent peaks of 4-hydroxybenzoic acid, hippuric acid, 4-hydroxyhippuric acid, and 3-methoxy-4-hydroxyhippuric acid were all identified under negative mode ionization at m/z 178.0511, 194.046, 224.0565, and 137.0245, respectively, in both blank urine and with marked increased S/N ratios at 8 A.M. in clinical urine samples. Pietta et al. also documented that vanillic acid, which they considered specific for ginkgo biloba metabolomics should be absent in blank urine [74]. However, it was detected in blank urine by the present method with a low S/N ratio at m/z 167.0351. We concur that its peak presence was either due to an overlap of an endogenous urine metabolite at the same m/z value, such as 2,6-dihydroxyphenylacetate (from phenylalanine metabolism) or homogentisate (from tyrosine metabolism). The peak S/N ratio increased 15 fold after multivitamin ingestion, leading us to concur that it is due to vanillic acid appearance. Additionally, a new signal corresponding to the deprotonated molecular peak of 3,4-dihydroxybenzoic acid was detected at m/z 153.0194. These results are depicted in Fig. 3.

Conclusion

This work builds on the advantages of the new DI-nano-ESI-MS/MS application for efficient analysis and the easiness and versatility of use of NS capillaries to provide a promising methodology for rapid and sensitive screening of dietary supplement ingredients. Because of its excellent efficiency, DI-nano-ESI-MS/MS was applied for targeted screening of water soluble vitamins, fat soluble vitamins, amino acids, ginkgo biloba, ginseng and royal jelly in multivitamins capsule in minimal time. Moreover, the tested analytes, as well as their related metabolites from complex sample matrix, urine, were detected by performing a dilute-and shoot procedure without any additional processing to verify the feasibility of the developed method. This approach is entirely innovative and overcomes the major difficulties associated with the conventional LC-MS techniques on simultaneous determination of multivitamins in human urine after oral administration of multivitamins capsule. Based on combination of off-line nano-ESI and MS/MS, a new DI-nano-ESI-MS/MS screening technique can be applied for index marker and subspecies identification as well as detection of chemical contaminants in dietary supplement safety analysis.

Compliance with Ethics Requirements

The study was conducted after approval by the ethical committee of the faculty of pharmacy Suez Canal University (approve number: 201901R2).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2020.06.009>.

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