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Rapid Simultaneous Determination of Four Indole Compounds in Dietary Supplements by Micellar Electrokinetic Chromatography with Dilute and Shoot Step

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1 **Rapid Simultaneous Determination of Four Indole Compounds in Dietary Supplements by**

2 **Micellar Electrokinetic Chromatography with Dilute and Shoot Step**

3

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25 ## Abstract

26

27 A simple micellar electrokinetic chromatography (MEKC) with UV detection was developed
28 for the simultaneous determination of indole-3-carbinol, indole-3-acetonitrile, indole-3-
29 acetic acid and 3,3´-diindolylmethane. These compounds are potentially used in cancer
30 prevention. Investigation of solvent effects (methanol and dimethylformamide) to MEKC
31 analysis was carried out. A dilute and shoot strategy was used for sample preparation to
32 reduce the time required for multiple steps such as solvent evaporation. The final conditions
33 were electrokinetic injection for 3.0 s at 423 V cm^{-1}, 20.0 mM borate buffer (pH 9.00),
34 containing 20.0 mM SDS. Analysis was rapid, achieved in less than 4.5 min. Linear calibration
35 curves for the indole compounds in the range 5–200 µg mL^{-1} (r^2> 0.999) were obtained.
36 Intra- and inter- day precisions were 5.1–7.9 %RSD, with LOQs of 1.5–4.0 µg mL^{-1} and
37 recoveries of 90–110% (n=5).

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39 *Keywords:* indole compounds, micellar electrokinetic chromatography, dietary supplement,
40 dilute and shoot

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1. Introduction

Indole compounds are one of the major components of *Brassica* vegetables, e.g. broccoli, cauliflower, cabbage and Brussels sprouts.[1-3] These natural indole compounds such as indole-3-carbinol (I3C), indole-3-acetonitrile (I3A), indole-3-acetic acid(IAA) and 3,3´-diindolylmethane (DIM) are of interest as promising preventive agents for cancers, such as breast, prostate and colon.[4, 5] As reported by Rogan, I3C is a hydrolysis product of glucobrassicin and is metabolized to a variety of I3C compounds, including I3A and DIM through the myrosinase enzyme activity.[6] The National Research Council Committee on Diet, Nutrition, and Cancer has noted the decreasing incidence of cancer with increasing consumption of *Brassica* vegetables.[7] Significant amount of indole in *Brassica* has been extracted to manufacture dietary supplement products.[8, 9] These products are available in health food stores, pharmacies and on-line shopping websites in many countries, especially USA and Europe.[10] Under the FDA guideline for labeling of dietary supplement product, the amount of ingredient/nutrition can be claimed with no conventional/standard method for analysis.[11] Analysis of indole compounds (from vegetables) have been reported using spectrophotometry,[12] gas chromatography (GC),[13, 14] and high-performance liquid chromatography (HPLC).[15, 16] However tedious sample preparation steps, such as extraction and evaporation were required prior to HPLC analysis.[3] GC analysis required derivatization of the indoles which was not convenient if rapid results are required.[13] Thus, development of a simple, rapid and reliable analytical method for the determination of I3C, DIM and related indole compounds in dietary supplements for quality control is needed, especially when there are a large number of samples for analysis.[17] Micellar electrokinetic

chromatography (MEKC) is a simple technique for separation of neutral compounds such as these indole compounds.[18] MEKC has been applied to the determination of some indole compounds in plants, broccoli and plant tissues with multi-step sample-pretreatment, such as liquid-liquid extraction and solid-phase extraction prior to the chromatography.[3, 19, 20] Dilute and shoot method is promising to incorporate into sample preparation since there is only a simple dilution of the aliquot of sample before direct measurement of the compounds.[21, 22] Simple dilute and shoot for MEKC analysis of these indole compounds in dietary supplements has not yet reported in the literature. This work is a rapid analysis of four indole compounds found in dietary supplements products by MEKC, with a dilute and shoot step for sample pretreatment (see Fig. 1).

2. Experimental

2.1 Chemicals and reagents

All indole standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecylsulfate (SDS) was from Merck (Darmstadt, Germany). Analytical grade dimethylformamide (DMF) and methanol was purchased from RCI Labscan (Bangkok, Thailand). All solutions were prepared and diluted to the desired concentrations using ultrapure water (18.0 MΩ cm^{-1}) from Easypure II system (Barnstead International, Iowa, USA).

2.2 Preparation of solutions

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3 95 The MEKC running buffer consisted of borate buffer (20.0 mM, pH 9.00) with SDS (20.0
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5 96 mM). Borate buffer was prepared from boric acid and adjusted to pH 9.00 with 1 M NaOH
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7 97 solution. Stock standard solutions of I3C, I3A, IAA and DIM (10 mg mL^{-1}) were prepared by
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9 98 accurately weighing 100 mg and dissolving with 1.00 mL DMF and making up to volume with
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11 99 borate buffer in a 10.00-mL volumetric flask. The standard solutions were kept in a
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13 100 refrigerator at 4 °C until needed. It can be stored with stability up to 3 months. Working
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15 101 standards (500 μg mL^{-1}) were prepared daily by dilution of stock standard with borate buffer
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17 102 solution. A calibration curve was constructed with concentrations of 5, 10, 25, 50, 100, and
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19 103 200 μg mL^{-1}, respectively, for each indole standard.
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23 105 **2.3 Instrumentation**
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27 107 The capillary electrophoresis system was assembled in-house. It consisted of a UV detector
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29 108 (Applied Biosystem, 785A UV detector, CA, USA), a high voltage (HV) power supply
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31 109 (Spellman CZE1000R, Hauppauge, USA) and a tray for the samples and buffer vials. The
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33 110 instrument was housed in a Plexiglas box with a micro switch to shutdown the high voltage
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35 111 power supply whenever the door of the box was opened. The absorbance signal was
36
37 112 recorded by a data acquisition system from eDAQ (Denistone East, NSW, Australia).
38
39 113 Measurement of electrophoretic current across the capillary column was recorded with the
40
41 114 same eDAQ system. A fused-silica capillary (50 µm i.d., 360 µm o.d.) was from Polymicro
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43 115 Technologies (Phoenix, AZ, USA). The total length of the capillary was 59.0 cm, with an
44
45 116 effective length of 38.0 cm from injection end to detector. The capillary column was
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47 117 conditioned before use by rinsing with a series of NaOH solution (0.1 M), ultra-pure water
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49 118 and borate buffer, using a spring loaded syringe (Unimicro Technologies, CA, USA).
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119 Electrokinetic injection for 3.0 s at 25 kV was used for sample introduction and detection
120 was at 280 nm.
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122 **2.4 Sample preparation**

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124 Samples of commercial dietary supplement were purchased from different suppliers (USA).
125 Three capsules were selected from each product and ground on a mortar and pestle. The
126 powder was then accurately weighed (30 mg) and dissolved in 5.00 mL DMF. The solution
127 was mixed thoroughly on a vortex mixer and sonicated for 5 min. The sample aliquot was
128 then filtered through a filter disk (0.45 μm cellulose acetate) and then diluted with borate
129 buffer solution (dilution factor of 1:20 for Samples A, C, D and 1:40 for Samples B and E).
130 Three sample aliquots were analyzed for each dietary supplement samples.
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132 **2.5 Method validation**

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134 Validation parameters, such as linearity, range, intra-day and inter-day precision, and
135 accuracy were investigated, following the FDA Guideline.[23] Limit of detection (LOD) and
136 quantification (LOQ) followed the ICH Guideline.[24] Mixture of I3C, I3A, IAA and DIM
137 standards at concentrations of 5, 10, 25, 50, 100, and 200 μg mL^{-1}, respectively, were
138 prepared to construct calibration curves. Each concentration was injected five times.
139 Precision (intra-, inter-day) of the method were determined using three aliquots at
140 concentrations of 25, 100, and 200 μg $mL^{-1}$of the standard mixtures, respectively, with five
141 replicate injections. The limit of detection (LOD) and limit of quantification (LOQ) were
142 calculated from the standard deviation (σ) of intercept and slope (s) of the calibration curve,

with $\text{LOD} = 3.3\sigma/s$ and $\text{LOQ} = 10\sigma/s$. The percentage sample recovery was calculated from $\%\text{Recovery} = \frac{S_1 - S_2}{S_0} \times 100$, where S_0 is peak area of pure standard solution, S_1 is peak area of spiked sample, S_2 is peak area of non-spiked sample. The concentration of each standard indole compounds was 100 µg mL^{-1}.

3. Results and discussion

3.1 Investigation of MEKC conditions

MEKC is an effective method for separation of neutral compounds in CE. MEKC is carried out by addition of SDS, at concentration above its critical micelle concentration (CMC), into the borate running buffer, resulting in dynamic partitioning of the analyte in the micellar pseudostationary phase.[25] Standard solutions of mixture of I3C, I3A, IAA and DIM were used to investigate MEKC conditions, using efficiency, resolution and analysis time as target parameters to select the final conditions.

3.1.1 Effect of buffer concentration

Fig. 2A shows electropherograms of a standard solution of indole compounds with various concentrations (20.0, 40.0 and 60.0 mM) of the borate buffer (pH 9.00), containing 20.0 mM SDS. Increasing the borate buffer concentration from 20.0 mM to 40.0 mM leads to a significant reduction in the signal for DIM but with no change in the resolution of the indole compounds. DIM has higher molecular weight, and thus larger in size when compared to the

other indoles (see Fig. 1). It has a lower electrophoretic mobility when compared to I3C, I3A and IAA. In addition DIM is less polar, and leads to better hydrophobic interaction with SDS micelle. Therefore DIM signal was more affected than the other indole compounds. The 60.0 mM borate buffer gave high electrophoretic current (~50 μA), leading to loss of separation of indole compounds (bottom electropherogram, Fig. 2A). Therefore 20.0 mM borate buffer was selected as the background electrolyte because high separation efficiency was obtained.

3.1.2 Effect of buffer pH

Fig. 2B shows the effect of pH (8.00, 8.50, 9.00 and 9.50) on the migration times of the four indole compounds. At pH 8.00, the peaks for I3A and IAA were not baseline separated (top electropherogram, Fig. 2B). Generally in CE, the lower the pH the smaller the EOF velocity but this is not the case in MEKC.[26] There were only small changes in the migration times of the indole compounds with pH, but there were significant effects on the peak widths, with DIM being most affected (see Fig. 2B, pH 8.00 and pH 8.50). This was due to the fact that indole compounds (I3C, I3A) are neutral and not affected in MEKC by the change of pH of the running buffers.[26, 31] IAA is a weak acid with pK_a of 4.75,[20] and is thus fully ionized at the pH range of the buffer (8.00 – 9.50). In the case of DIM, it strongly interacts with SDS micelle due to its hydrophobicity. The buffer at pH 9.00 provided the best peak resolutions (R_s = 2.08 for I3A peak and IAA peak) and the greatest peak intensities.

3.1.3 Effect of SDS concentration

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190 Fig. 2C shows the effect of SDS concentrations (0, 20.0, 40.0 and 60.0 mM) on the
191 electropherograms. The concentrations are all above the CMC of SDS. As shown in Fig. 2C,
192 the neutral indole compounds are, as expected, not separated without addition of SDS.
193 However the SDS concentration greatly affected separation efficiency and peak
194 intensities.[27, 28] A higher number of micelles results from a higher SDS concentration and
195 suitable SDS concentration should be tested for optimal separation.[28] SDS concentration at
196 20.0 mM was selected as the optimal concentration in the borate running buffer because of
197 its separation efficiency (Fig. 2C, 20.0 mM). In this study addition of individual indole
198 standard to the sample solution was used to identify the peaks. Stable EOF was checked by
199 monitoring electrical current before investigation of SDS concentration effect. Lower
200 resolution between I3A and IAA was observed when using 40.0 mM SDS while DIM peak
201 broadening was found when using 40.0 mM and 60.0 mM SDS (see Fig. 2C). This was due to
202 high concentration of SDS leading to larger retention of analytes in the micelles.[25]
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204 **3.1.4 Effect of injection time**
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206 Sample introduction for our in-house CE system was by electrokinetic injection. A constant
207 field strength of 423 V cm^{-1} was applied with varying injection times of 1.5 s, 3.0 s, 5.0 s and
208 10.0 s, respectively, measured using a digital timer (TA228, Shenzhen Liweihui Technology
209 Co., Ltd.) with precision of 100 ms. A standard solution (100 µg mL^{-1} of the 4 indoles) was
210 used. As expected, the longer the injection time the higher was the peak width at half
211 maximum height: the peak widths were in the range of 2.3±0.1 s, 2.9±0.1 s, 5.3±0.2 s and
212 10.7±0.7 s, for injection times of 1.5 s, 3.0 s, 5.0 s and 10.0 s, respectively. The shortest
213 injection time (1.5 s) gave precision of the peak area of 7.8 %RSD. Injection times of 3.0 s

and 5.0 s had comparable precisions of 2.7% and 3.1 %RSD, respectively. However at a longer injection time (10.0 s), the %RSD increased to 6.4%. Injection time of 3.0 s was selected as the operating condition.

3.1.5 Precision of EOF velocity

The electroosmotic flow velocity was monitored to evaluate the stability of MEKC system. The final MEKC conditions were; 20.0 mM borate buffer (pH 9.00) containing 20.0 mM SDS, electrokinetic injection of 3.0 s at 423 V cm^{-1}. The EOF velocity was obtained from measurement of the time required to completely replace the capillary buffer at a lower concentration (20.0 mM) with one at a higher concentration (40.0 mM) (or vice versa), by monitoring the electrical current.[29] EOF velocity, v_{eof} was calculated from $v_{eof} = \frac{L_d}{t_m}$; where L_d is the effective length of capillary column (38.0 cm), and t_m is the measured migration time. Inter-day precision was calculated from five replicates at five different days using the same operating conditions. The EOF velocity was 7.68±0.26 cm min^{-1} (3.4 %RSD) showing the high degree of stability of the MEKC system. The EOF was stable up to 15 consecutive injections (~1.2 hrs), before next rinsing required for capillary conditioning. Fig. 3A shows high peak resolution of the electropherogram of I3C, I3A, IAA and DIM, at concentration of 100 µg mL^{-1}.

3.2 Method validation

The validation data of the proposed method are shown in Table 1. Calibration curves were linear over the concentration range of 5–200 µg mL^{-1} for I3C, I3A, IAA and DIM, with r^2>

0.999 for all indole compounds. The limit of detection (LOD) was 0.5–1.3 µg mL^{-1}, with limit of quantification (LOQs) of 1.5–4.0 µg mL^{-1}. Accuracy of the determination was evaluated from percentage recovery of sample. Mixture of I3C and DIM standards solution (100 µg mL^{-1} each) was added into the sample aliquot (n=3) before MEKC analysis. Percentage recovery $\left(\%\text{Recovery} = \frac{S_1 - S_2}{S_0} \times 100\right)$ was calculated, where S_0 is peak area of pure standard solution, S_1 is peak area of spiked sample, S_2 is peak area of non-spiked sample. The recovery data of the I3C and DIM compounds ranged from 90–110% for the five samples (Table 1), with %RSD range 4–11% (n=3). The intra-day and inter-day precisions were 2.0–5.2 %RSD and 2.2–7.9 %RSD, respectively (Table 2). The %RSD values were within the acceptable limit of < 15 %RSD.[23]

3.3 Effect of solvent on MEKC analysis

Dissolving of indoles dietary supplement can be carried out using an organic solvent before diluting with buffer medium. This study investigated effect of methanol and DMF as dissolution solvent on MEKC analysis when applying dilute and shoot stragegy.[3, 30] A series of standard mixture solutions (50 to 200 µg mL^{-1}) of all four indole compounds were used to investigate effect of dilution with different percentage of methanol and DMF present, as presented in Fig. 4A and Fig. 4B, respectively. Percentage of each solvent in the borate buffer was varied from 1% to 20% (%v/v). The slope (sensitivity) for each percentage of solvent was determined and normalized against 0.05% DMF to compare the effect of different amounts of solvent (1% to 20% methanol (Fig. 4A) and 1% to 20% DMF (Fig.4B)). Significant changes in the slopes were observed when the percent of solvent was above 5%, especially for methanol. Fig. 4A shows that methanol affected the sensitivity of indoles

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3 262 especially IAA and DIM, whereas DMF solvent gave smaller variation as seen in Fig. 4B. For
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5 263 1% to 5% DMF the change in slopes of I3C, I3A, IAA and DIM were not significantly different
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7 264 (≤ 8%). Changes were higher when DMF of 10% and 20% were used. Therefore 5% DMF was
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9 265 selected for dilution of the dietary supplement sample.
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13 267 **3.4 Dilute and shoot with MEKC analysis of dietary supplement products**
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17 269 Theindole contents of five dietary supplements were determined using the developed MEKC
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19 270 method. The indoles dietary supplements were commercially available and labeled as
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21 271 containing I3C, DIM and related indole compounds. It should be noted that the sample,
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23 272 dissolved in DMF, was diluted with borate buffer and directly injected without prior
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25 273 evaporation of the solvent (dilute and shoot step). Electrophoretograms of a mixture of the
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27 274 four indole standards and two representative samples are shown in Figs. 3A, 3B, and 3C,
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29 275 respectively. Table 3 lists the measured amount of indole related compounds, in mg per
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31 276 capsule of dietary supplement products; for samples A, B, C, D and E, %RSD of the weight of
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33 277 the sample powder per capsule was in range of 2–6% for all products. Typically the label
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35 278 amount of indole dietary supplement is given as the sum of the contents of I3C, DIM and
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37 279 related indole compounds. The sums of all the measured indole compounds were compared
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39 280 with the label values and were comparable with the label amounts for all samples;
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41 281 percentage difference was lower than 9%.
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45 283 **4. Conclusions**
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A MEKC method for analysis of indole compounds (I3C, I3A, IAA and DIM) was developed, using an in-house capillary electrophoresis system with UV detection. Determination of dietary indole supplement products were applied with dilute and shoot method. This was a fast and easy sample preparation step prior to MEKC analysis. MEKC conditions were investigated in terms of buffer concentration and pH, SDS concentration and injection time. This is the first report of simultaneous analysis of four indole compounds by a simple MEKC method. The MEKC analysis was much faster (less than 5 min) than previous chromatographic methods which required an hour of analysis time.[13, 15] Our MEKC method also provides advantages in terms of wider dynamic range, comparable precision with HPLC/GC analysis, consumption of smaller volume of reagent (nL to μL), with very simple method for sample pretreatment (dilute and shoot procedure). This MEKC method is not only a method for monitoring the quality of indole containing dietary supplements, but it may be applicable for determination of indoles in other types of samples, such as cruciferous vegetables, urine or blood, for which a pre-concentration step may be required.

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373 **List of Tables**

374

375 **Table 1** Validation data of the MEKC method; regression equation, coefficient of
376 determination (r^2), linear range, limit of detection (LOD), limit of quantification (LOQ) and
377 percentage recovery of sample

Analyte	Regression equation	Coefficient of determination (r^2)	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	%Recovery
I3C	y = 3.064x-1.337	0.9998	5-200	0.7	2.3	90-110
I3A	y = 6.002x-12.461	0.9990	5-200	0.5	1.5	NA
IAA	y = 3.894x+0.160	0.9993	5-200	0.5	1.6	NA
DIM	y = 5.366x+1.252	0.9995	5-200	1.3	4.0	90-105

378 *NA (Not applicable). These indole compounds were not detected in all dietary supplement
379 samples analyzed.

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Table 2 Inter-day and intra-day precisions of the MEKC method

Indole compound	Concentration ($\mu g\ mL^{-1}$)	Precision (%RSD), n=5	
		Intra-day	Inter-day
I3C	high	2.0	5.0
	medium	2.1	5.1
	low	4.1	2.2
I3A	high	4.5	2.9
	medium	4.9	4.7
	low	5.2	3.9
IAA	high	4.5	4.6
	medium	3.2	4.0
	low	4.8	3.9
DIM	high	4.2	7.9
	medium	3.9	5.1
	low	5.1	7.3

%RSD; percentage of relative standard deviation

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391 **Table 3** Determination of indole compounds in the dietary supplement samples

Sample	Measured content mg/capsule				Sum mg/capsule	Label amount of total indole compounds mg/capsule
	I3C±SD	I3A±SD	IAA±SD	DIM±SD		
A	135±3	ND*	ND*	290±15	425	400
B	210±10	ND*	ND*	ND*	210	200
C	140±7	ND*	ND*	47±4	187	200
D	85±5	ND*	ND*	7±1	92	100
E	167±2	ND*	ND*	25±1	192	200

392 *ND (Not detected)

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3 399 **Lists of Figure Caption**
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8 401 **Fig. 1** Chemical structure of indole compounds. (A) Indole-3-carbinol, (B) Indole-3-
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10 402 acetonitrile, (C) Indole-3-acetic acid, and (D) 3,3′-Diindolylmethane.
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15 404 **Fig. 2** Electropherograms of standard mixture solutions of indole compounds (100 µg mL^{-1} of
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17 405 I3C, I3A, IAA, DIM); (A) Effect of borate buffer concentrations of 20.0 mM, 40.0 mM, and
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19 406 60.0 mM, each buffer containing SDS of 20.0 mM at pH 9.00; (B) Effect pH of borate buffer
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21 407 (8.00, 8.50, 9.00, 9.50) each containing SDS of 20.0 mM and(C) Effect of SDS concentrations
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23 408 adding to borate buffer solution (20.0 mM, pH 9.00). The SDS concentrations at 0 mM, 20.0
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25 409 mM, 40.0 mM and 60.0 mM were varied. The running MEKC conditions used were as
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27 410 follows: electrokinetic injection for 3.0 s at 423 V cm^{-1}, applied electrical field strength of
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29 411 423 V cm^{-1} for separation, and UV detection at 280 nm. *Unidentified peak.
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32 412
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34 413 **Fig. 3** Electropherograms of (A) standard mixture solution of indole compounds (I3C, I3A,
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36 414 IAA, DIM); dietary supplement samples, (B) Sample A (20x dilution factor) and (C) Sample B
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38 415 (40x dilution factor). MEKC conditions were: borate buffer (20.0 mM, pH 9.00) containing
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40 416 20.0 mM SDS, electrokinetic injection for 3.0 s at 423 V cm^{-1}, applied electrical field strength
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42 417 of 423 V cm^{-1} for separation, and UV detection at 280 nm. *Unidentified peak.
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46 419 **Fig. 4** The normalized plot of slope of a linear curve of standard indole compounds with
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48 420 different percentage of (A) methanol and (B) DMF in the borate buffer solution (% v/v).
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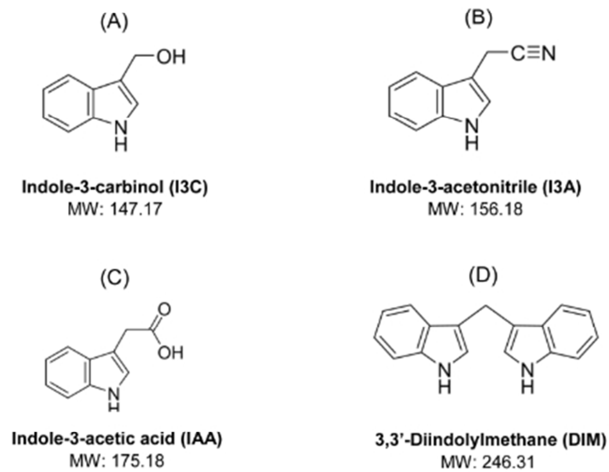

Fig. 1

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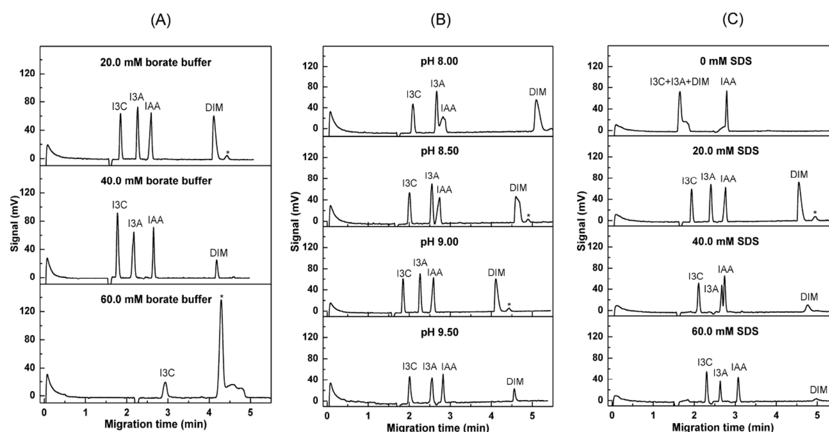

Fig. 2

209x148mm (300 x 300 DPI)

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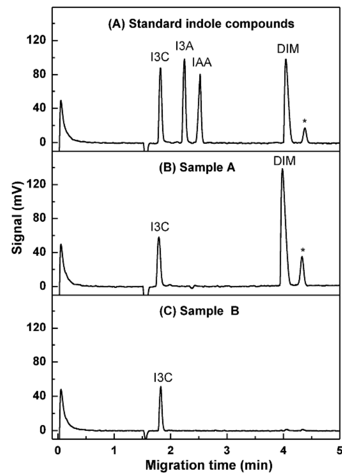

Fig. 3

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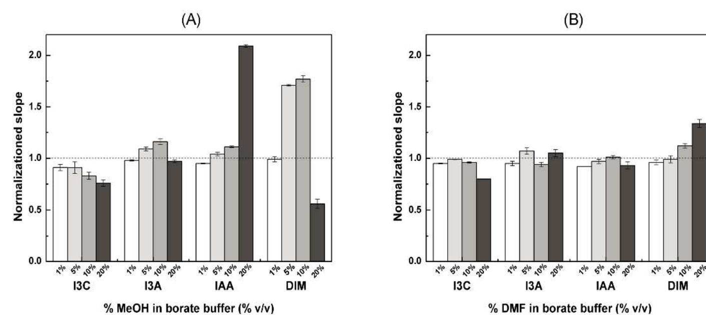

Fig. 4

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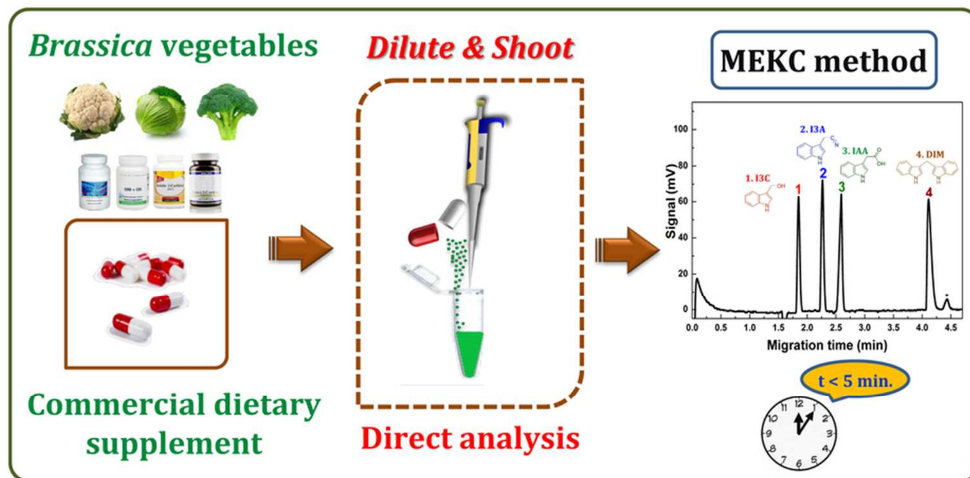

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