A rapid and simple capillary electrophoresis procedure for quantification of vanillylmandelic acid in urine samples

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SUMMARY

**Aim:** To develop a one-step vortex-assisted liquid-liquid extraction (VALLE) method, without the need for evaporation and reconstitution steps, to establish a rapid and straightforward treatment procedure based on capillary electrophoresis-diode array detection (CE-DAD) for the determination of vanillylmandelic acid (VMA) in human urine. **Methodology:** Optimization of VALLE and CE-DAD procedures were studied in detail. The effects of various experimental parameters, such as the type of the extraction solvent, sample pH, salt addition, and extraction time were investigated. Also, CE separation conditions including background electrolyte type, concentration, and pH, injection time and separation voltage were optimized as well. **Results:** A successful separation of VMA was achieved in less than 6 min using a basic background electrolyte composed of 60 mmol·L⁻¹ acetate/ACN (acetonitrile) 50% (v/v) (final apparent pH is 5.73). The linear response was obtained over the concentration range from 1.0 to 14 µg·mL⁻¹. The limit of detection and quantification were 0.30 and 1.0 µg·mL⁻¹, respectively. The intra- and inter-day precisions were found to be less than 4.3%. The extraction recoveries of VMA were between 95%-97%. **Conclusion:** The developed method is found to be a simple, rapid, and reliable method for quantitative analysis of urinary VMA.

**Keywords:** Extraction recovery, capillary electrophoresis, diode-array detection, urine, vortex-assisted liquid-liquid extraction.
Un procedimiento de electroforesis capilar rápido y simple para la cuantificación de ácido vanililmandélico en muestras de orina

**Objetivo:** desarrollar un método de extracción líquido-líquido asistido por vórtice (MELLAV) de un solo paso, sin la necesidad de pasos de evaporación y reconstitución, para establecer un procedimiento de tratamiento rápido y sencillo basado en electroforesis capilar con detección por arreglo de diodos (EC-DAD) para la determinación de ácido vanililmandélico (AVM) en orina humana. **Metodología:** se estudió en detalle la optimización de los procedimientos MELLAV y EC-DAD. Se investigaron los efectos de varios parámetros experimentales, como el tipo de solvente de extracción, el pH de la muestra, la adición de sal y el tiempo de extracción. Además, también se optimizaron las condiciones de separación EC, incluyendo el tipo de electrolito de fondo, la concentración y el pH, el tiempo de inyección y el voltaje de separación. **Resultados:** se logró una separación exitosa de AVM en menos de 6 min utilizando un electrolito de fondo básico compuesto por 60 mmol-L⁻¹ de acetato/ACN (acetonitrilo) al 50 % (v/v) (el pH aparente final es 5,73). La respuesta lineal se obtuvo en el rango de concentración de 1,0 a 14 µg·mL⁻¹. El límite de detección y cuantificación fue de 0,30 a 1,0 µg·mL⁻¹, respectivamente. Se encontró que las precisiones intra e interdiarias eran inferiores al 4,3 %. Las recuperaciones de extracción de AVM estuvieron entre 95%-97%. **Conclusión:** el método desarrollado resulta ser un método simple, rápido y confiable para el análisis cuantitativo de AVM en orina.

**Palabras clave:** Recuperación por extracción, electroforesis capilar, detección por arreglo de diodos, orina, extracción líquido-líquido asistida por vortex.

Um procedimento rápido e simples de eletroforese capilar para a quantificação de ácido vanilmandélico em amostras de urina

**Objetivo:** desenvolver um método de extração líquido-líquido assistido por vórtice (MELLAV) em uma etapa, sem a necessidade de etapas de evaporação e reconstituição, estabelecer um procedimento de tratamento rápido e simples baseado em eletroforese capilar com detecção de arranjo de diodos (EC-DAD) para a determinação de ácido vanilmandélico (VMA) na urina humana. **Metodologia:** a otimização dos procedi-
mentos MELLAV e EC-DAD foi estudada em detalhes. Os efeitos de vários parâmetros experimentais, como o tipo de solvente de extração, pH da amostra, adição de sal e tempo de extração, foram investigados. Além disso, as condições de separação EC também foram otimizadas, incluindo o tipo de eletrólito de fundo, concentração e pH, tempo de injeção e tensão de separação. Resultados: uma separação AVM bem-sucedida foi obtida em menos de 6 min usando um eletrólito de fundo básico composto de 60 µg·mL⁻¹ 50% (v/v) de acetato/ACN (acetonitrila) (pH final aparente é 5,73). A resposta linear foi obtida na faixa de concentração de 1,0 a 14 µg·mL⁻¹. O limite de detecção e quantificação foi de 0,30 a 1,0 µg·mL⁻¹, respectivamente. As precisions intradias e interdiass foram inferiores a 4,3%. As recuperações de extração de AVM ficaram entre 95%-97%. Conclusão: o método desenvolvido revela-se um método simples, rápido e confiável para a análise quantitativa de MAV em urina.

Palavras-chave: Recuperação de extração, eletroforese capilar, detecção de matriz de diodos, urina, extração líquido-líquido assistida por vórtice.

**INTRODUCTION**

Vanillylmandelic acid (VMA, DL-4-hydroxy-3-methoxybenzeneacetic acid with a molar mass of 198.2 g·mole⁻¹, for the chemical structure of VMA, see figure 1) is the final product of epinephrine and norepinephrine metabolism in the human body [1]. It is well known that the concentration of VMA, as a well-established biomarker, increases in various diseases such as autism [2], neuroblastoma [3], pheochromocytoma [4], and tumors of the neural crest [5], while it may decrease in situations such as depressions [6]. Due to the easy and non-invasive collection, urine samples are frequently used to determine VMA in clinical diagnosis [7]. Normal concentrations of VMA in healthy individuals vary over a wide range in the urine based on the values described in the bibliography, that is from 11.6 to 28.7 µmol·L⁻¹ (or 2.3 to 5.7 µg·mL⁻¹) [8].

![Chemical structure of vanillylmandelic acid (VMA)](image)

**Figure 1.** Chemical structure of vanillylmandelic acid (VMA).
Several analytical separation techniques, namely high-performance liquid chromatography (HPLC) with either electrochemical detection (ECD) [9-12], fluorescence detection (FLD) [13-16] or mass spectrometry (MS) [17-24] and gas chromatography coupled to mass spectrometry (GC-MS) [25-27] or flame ionization detector [28] have been reported for the determination of VMA. HPLC-ECD needs laborious sample preparation and extensive analytical time [19]. Although HPLC-tandem mass spectrometry (HPLC-MS/MS) possesses improved selectivity and sensitivity and decrease analysis time, it is costly and needs a qualified and skilled operator. GC-MS offers high identification and separation power, but for most of analytes, it requires a time-consuming derivatization procedure to become volatile, stable, and amenable to the ionization techniques [29]. The use of immunoassay techniques [30, 31] and electrochemical methods [32-34] has also been described in the literature. However, the selectivity and specificity of separation techniques are higher than immunoassay and electrochemical methods, especially in complex biological samples. Capillary electrophoresis (CE) in different separation modes provides superior separation efficiency, short analysis time, small sample and low reagent consumption, and automatability that make it an attractive alternative to HPLC [35]. Therefore, a growing interest in the application of CE to VMA analysis in urine samples can be observed [8, 36-41].

Before the application of the separation technique during quantitative analysis, the sample preparation step is usually needed to be developed and optimized for the selective extraction of analytes of interest from biological samples and to clean-up the sample matrix. Solid-phase extraction (SPE) [42-44] and liquid-liquid extraction (LLE) [38, 41, 45] methods are usually used to pre-treatment urine samples prior to the analysis of VMA. SPE is an efficient sample preparation technique but requires proper sorbent materials and solvents, as well as multiple washing and elution steps, which is tedious and labor-intensive [46]. On the other hand, LLE due to its simplicity and efficiency was described as a good alternative to SPE. Simpler methods such as dilution, centrifugation, and filtration either alone or in combination have also been reported to clean-up urine samples prior to injection to CE [8, 36, 39, 40], but the resulting electropherograms possess many peaks that make it difficult to identify the analyte of interest and also reduce the selectivity of the method. These methods do not eliminate the interferences. In addition, under the influence of the contents of the sample matrix, the performance of the separation column is reduced. Therefore, in the case of CE analysis of VMA, vortex-assisted LLE (VALLE) is often used for the treatment of urine samples [38, 41]. However, VALLE requires evaporation and reconstitution steps, and hence additional time. Furthermore, due to the possibility of analyte
loss in these steps, it is necessary to use a suitable internal standard. The main analytical methods for the determination of VMA in urine along with sample preparation are summarized in table 1.

To diagnose of aforementioned diseases and to monitor response to treatment, the demand for the routine determination of VMA is increasing. Therefore, low-cost and rapid methods are necessary to satisfy the rising demand. CE is a rapid and cost-effective method compared to other mentioned methods. However, using the conventional VALLE method for sample preparation can be a source of error and is time-consuming [8]. In conventional capillary zone electrophoresis (CZE) with aqueous running buffer, direct injection of organic solvent disrupts the separation process due to its very low conductivity. But, the addition of organic solvents to the running buffer composed of a binary solvent allows direct injection of the sample extracted in organic solvents [47]. Therefore, the main goal of the present study was to develop the rapid and simple VALLE method for extraction of VMA from urine samples without the need for additional time-consuming and error-prone evaporation and reconstitution steps. For this purpose, several organic solvents were tested for effective extraction of VMA. Subsequently, the extraction solvent (upper phase, supernatant) was taken and directly injected onto CE-diode array detection (CE-DAD). The method presented here is fast, straightforward, and reliable.
Table 1. A review of main analytical methods for the measurement of VMA in urine.

<table>
<thead>
<tr>
<th>Analysis method</th>
<th>Sample treatment /reagent</th>
<th>Sample treatment time (min)</th>
<th>Extraction recovery (%)</th>
<th>LOD (µg·mL⁻¹)</th>
<th>LOQ (µg·mL⁻¹)</th>
<th>LR (µg·mL⁻¹)</th>
<th>Method imprecision (RSD %)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC-FLD</td>
<td>LLE/ ethyl acetate; agitation by shaker</td>
<td>&gt; 60</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>5.0-20</td>
<td>4.0</td>
<td>[13]</td>
</tr>
<tr>
<td>HPLC-E-CD</td>
<td>LLE/ ethyl acetate; agitation by shaker</td>
<td>50-80</td>
<td>72</td>
<td>0.81</td>
<td>2.0</td>
<td>2.0 ¹⁵</td>
<td>≤6.0</td>
<td>[11]</td>
</tr>
<tr>
<td>HPLC-FLD</td>
<td>SPE</td>
<td>&gt; 20</td>
<td>98.3</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
<td>7.9</td>
<td>[15]</td>
</tr>
<tr>
<td>HPLC-E-CD</td>
<td>LLE/ ethyl acetate; agitation by shaker</td>
<td>&gt; 30</td>
<td>70</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.5</td>
<td>[12]</td>
</tr>
<tr>
<td>HPLC-FLD</td>
<td>LLE/ diethyl ether; agitation by vortex</td>
<td>&gt; 20</td>
<td>-</td>
<td>0.50</td>
<td>0.20</td>
<td>0.20-99</td>
<td>6.7</td>
<td>[14]</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>Centrifugation and dilution (10 times)</td>
<td>&lt;5</td>
<td>-</td>
<td>0.001</td>
<td>0.005</td>
<td>0-50</td>
<td>≤14.6</td>
<td>[22]</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>Dilution (50 times) and filtration</td>
<td>-</td>
<td>-</td>
<td>0.0002</td>
<td>0.001</td>
<td>0.001 ¹⁰</td>
<td>4.3</td>
<td>[23]</td>
</tr>
<tr>
<td>UPLC-MS/MS</td>
<td>Dilution (50 times)</td>
<td>0.117</td>
<td>0.355</td>
<td>0.25 ¹⁰⁰</td>
<td>≤7.0</td>
<td></td>
<td></td>
<td>[18]</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>Microextraction by packed sorbent (MEPS)/ eVol²</td>
<td>57.5–70.5</td>
<td>-</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50 ¹⁰⁰</td>
<td>≤9.6</td>
<td>[17]</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>Dilution (10 times) and centrifugation</td>
<td>-</td>
<td>-</td>
<td>0.18</td>
<td>0.50</td>
<td>0.50 ¹⁰⁰</td>
<td>≤4.0</td>
<td>[20]</td>
</tr>
<tr>
<td>HPLC-E-CD/UV</td>
<td>SPE</td>
<td>-</td>
<td>100</td>
<td>0.38 (ECD)</td>
<td>2.0 (ECD and UV)</td>
<td>2.0-30 (ECD and UV)</td>
<td>16 (ECD) ³</td>
<td>[10]</td>
</tr>
</tbody>
</table>

² eVol: exact volume
<table>
<thead>
<tr>
<th>Analysis method</th>
<th>Sample treatment /reagent</th>
<th>Sample treatment time (min)</th>
<th>Extraction recovery (%)</th>
<th>LOD (µg·mL⁻¹)</th>
<th>LOQ (µg·mL⁻¹)</th>
<th>LR (µg·mL⁻¹)</th>
<th>Method imprecision (RSD %)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC-MS/MS</td>
<td>Centrifugation and dilution (10 times)</td>
<td>&lt;5</td>
<td>-</td>
<td>0.005</td>
<td>0.025</td>
<td>0.20-50</td>
<td>≤3.12</td>
<td>[19]</td>
</tr>
<tr>
<td>UPLC-FLD</td>
<td>Centrifugation, filtration and dilution (almost 6 times)</td>
<td>-</td>
<td>-</td>
<td>0.012</td>
<td>0.037</td>
<td>0.01-20</td>
<td>5.78</td>
<td>[16]</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>DLLME/ extraction solvent (dichloromethane) and disperser solvent (ethanol)</td>
<td>33</td>
<td>99</td>
<td>0.005</td>
<td>0.01</td>
<td>0.005-2</td>
<td>≤9.8</td>
<td>[24]</td>
</tr>
<tr>
<td>GC-FID</td>
<td>1) LLE/ethyl acetate and ether  &lt;br&gt;2) Derivatization by BSTFA/trimethyl-chlorosilane</td>
<td>&gt;50</td>
<td>79.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[28]</td>
</tr>
<tr>
<td>GC-MS</td>
<td>1) LLE/ ethyl acetate  &lt;br&gt;2) Derivatization by BSTFA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[27]</td>
</tr>
<tr>
<td>GC-MS</td>
<td>1) LLE/ mixture of ethyl acetate and diethyl ether  &lt;br&gt;2) Derivatization by BSTFA</td>
<td>40</td>
<td>-</td>
<td>0.15</td>
<td>0.45</td>
<td>1.0-50</td>
<td>≤9.9</td>
<td>[2]</td>
</tr>
<tr>
<td>GC-MS</td>
<td>1) LLE/ ethyl acetate  &lt;br&gt;2) Derivatization by BSTFA</td>
<td>&gt;75</td>
<td>-</td>
<td>-</td>
<td>0.18</td>
<td>-</td>
<td>9.0</td>
<td>[26]</td>
</tr>
<tr>
<td>Analysis method</td>
<td>Sample treatment /reagent</td>
<td>Sample treatment time (min)</td>
<td>Extraction recovery (%)</td>
<td>LOD (µg·mL⁻¹)</td>
<td>LOQ (µg·mL⁻¹)</td>
<td>LR (µg·mL⁻¹)</td>
<td>Method imprecision (RSD %)</td>
<td>Ref.</td>
</tr>
<tr>
<td>-----------------</td>
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<td>---------------------------</td>
<td>------</td>
</tr>
<tr>
<td>CE-DAD</td>
<td>Centrifugation and filtration</td>
<td>5</td>
<td>-</td>
<td>0.001</td>
<td>0.025</td>
<td>-</td>
<td>-</td>
<td>[36]</td>
</tr>
<tr>
<td>CE-ECD</td>
<td>LLE/ ether; agitation by vortex</td>
<td>-</td>
<td>-</td>
<td>0.16</td>
<td>1.0</td>
<td>1.0</td>
<td>100</td>
<td>4.9</td>
</tr>
<tr>
<td>CE-UV</td>
<td>PP/ 1 mM formic acid containing 20% ACN and dilution (10 times)</td>
<td>10</td>
<td>-</td>
<td>0.062</td>
<td>-</td>
<td>-</td>
<td>3.1</td>
<td>[39]</td>
</tr>
<tr>
<td>CE-ECD</td>
<td>Centrifugation, filtration and dilution</td>
<td>-</td>
<td>-</td>
<td>0.005</td>
<td>0.01</td>
<td>0.01-5.0</td>
<td>3.4</td>
<td>[40]</td>
</tr>
<tr>
<td>CE-DAD</td>
<td>LLE/ diethyl ether; agitation by vortex</td>
<td>&gt;20</td>
<td>96.1</td>
<td>0.10</td>
<td>0.50</td>
<td>0.50-50</td>
<td>≤9.1</td>
<td>[41]</td>
</tr>
<tr>
<td>CE-UV</td>
<td>Centrifugation and dilution (1 time)</td>
<td>5</td>
<td>-</td>
<td>0.006</td>
<td>0.60</td>
<td>0.60-19.5</td>
<td>7.0</td>
<td>[8]</td>
</tr>
</tbody>
</table>

Experimental

Chemicals and reagents
VMA (purity ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The reagents used for the preparation of buffer solutions and the samples such as hydrochloric acid (HCl), phosphoric acid, acetic acid, sodium hydroxide (NaOH), sodium chloride (NaCl), sodium dihydrophosphate, sodium tetraborate decahydrate (borax), diethyl ether, 1-octanol, ethyl acetate, acetonitrile (ACN), and methanol (MeOH) were from Merck (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification. Deionized (DI) water was supplied by Shahid Ghazi Pharmaceutical Company (Tabriz, Iran).

Preparation of stock and working solutions
The stock solution of VMA was prepared by accurately weighing 20.0 mg and dissolving in 100 mL of DI water which was stored in a dark container in a freezer (−20 °C). This solution was used for the preparation of calibration and quality control (QC) samples. The working solutions were prepared daily by diluting the stock solution as appropriate with pure water or urine.

Preparation of human urine samples
A human urine sample from a healthy volunteer was collected. The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (code of ethics committee: IR.TBZMED.REC.1400.335). VALLE was used to treatment of urine samples, for this purpose, the pH of 1 mL of urine was adjusted to pH 1.0 then 0.2 g NaCl was added and briefly vortexed to the dissolution of NaCl. Subsequently, 1 mL of diethyl ether was added, vortexed for 10 min and centrifuged at 3000 rpm for 5 min. An aliquot of 20 µL of the upper layer was transferred into a 1 mL conical microtube and 20 µL methanol was added and briefly vortexed then an aliquot of 20 µL of the homogenous solution was transferred into the CE microvial to be injected into the CE system through the autosampler.

Capillary electrophoresis instrument
CE separation was carried out using an Agilent CE system equipped with a diode array detector (DAD) (190-600 nm). Data acquisition and control were accomplished using ChemStation (Agilent Technologies, Waldbronn, Germany). Separations were performed on a 36 cm bare fused silica capillary with a 27.5 cm effective length and an internal diameter of 50 µm (Agilent Technology, Waldbronn, Germany). The capillary was preconditioned with 1 M NaOH for 30 min, followed by DI water for 30 min and
A capillary electrophoresis procedure for quantification of vanillylmandelic acid

background electrolyte (BGE) solution for 30 min. Between runs, the capillary was rinsed with 0.1 M NaOH for 2 min, DI water for 2 min, and BGE solution for 4 min. The BGE solution was 60 mM acetate/ACN (1:1, v/v) buffer with an apparent pH of 5.73. The injection was by pressure (50 mbar) for 3 s. The separations were carried out at +20 kV and the capillary temperature was thermostated at 25 °C. BGE solution was filtered through a 0.45 μm pore size PTFE syringe filter (Chromafil, Germany) and degassed using sonication prior to use. The detection wavelength was 200 nm. UV spectra (200–400 nm) were collected for VMA identification and to check its purity. Also, peak identity is confirmed by spiking of standard solution.

Calculations

Relative recovery (RR) or recovery is calculated as follow:

\[
RR\% = \left( \frac{C_{\text{found}} - C_{\text{real}}}{C_{\text{added}}} \right) \times 100
\]

(1)

where \(C_{\text{found}}\), \(C_{\text{real}}\), and \(C_{\text{added}}\) are the measured concentration of the analyte in the real sample after the spike of a known amount of the standard, the original concentration of the analyte present in the real sample, and the concentration of the standard spiked in the real sample, respectively. The extraction recovery (ER) indicates how much of the analyte is transferred from the sample solution to the extraction solvent at the end of extraction and is defined as the ratio of the total analyte amount, which was extracted in the organic phase:

\[
ER\% = \left( \frac{n_{\text{o,final}}}{n_{\text{s,initial}}} \right) \times 100 = \left( \frac{V_o}{V_s} \right) \left( \frac{C_{\text{o,final}}}{C_{\text{s,initial}}} \right) \times 100
\]

(2)

Where \(n_{\text{s,initial}}\), \(n_{\text{o,final}}\), \(V_o\), \(V_s\), \(C_{\text{o,final}}\) and \(C_{\text{s,initial}}\) are the amount of analyte originally present in the sample solution, the amount of analyte transferred to the organic phase, the volume of the organic phase, the volume of the sample solution, the concentration of analyte in the organic phase, and the concentration of analyte in the sample solution, respectively.

Results and discussion

This study has two main objectives. First, to optimize the effective and straightforward extraction protocol for VMA from urine samples. For this purpose, three extraction solvents were tested, and then the resultant organic phase was directly injected
onto CE for further analysis. The second concerned the compatibility of the selected extraction method with further determination by the use of CE-DAD. Therefore, the optimization of the simple, rapid, and most efficient extraction procedure with further CE-DAD determination method was carefully accomplished.

**Optimization of sample preparation**

Sample preparation is a crucial step during the analysis of biological samples such as urine. VALLE is used here to clean up the urine sample. In LLE, the analyte of interest is transferred from an aqueous matrix into an extraction solvent. The extraction efficiency of LLE is improved by the adjustment of ionic strength and pH in the aqueous phase. Since the VMA (pKa = 3.11 [48]) is ionized in the urine (pH>4), it needs to be in molecular form to increase the efficiency of extraction. Therefore, the extraction pH must be optimized. By adding salt to the sample, known as the salting-out effect, the ionic strength of the sample can be changed to increase the extraction efficiency. The contact time between the sample and extraction solvent is also effective. However, the most important option is to select the proper organic solvent in order to achieve maximum recovery. Based on the values reported [41] for extraction of VMA in the previous study, the initial values of 1 for pH, 10 min for vortex agitation, and 0.2 g for NaCl were considered. Then, the extraction yield of 1-octanol, diethyl ether, and ethyl acetate was investigated. Under these conditions, extraction efficiencies in terms of ER were obtained for 1-octanol, diethyl ether, and ethyl acetate 78%, 97%, and 91%, respectively. Diethyl ether was selected for the rest of the experiments. pH values of 1, 2, 4, and 7 were implemented to determine how urine pH affects the extraction efficiency. The maximum extraction yield was obtained in pH 1, at pH values above 4, the extraction yields of VMA were lower as expected. In acidic pH, almost all molecules are neutral, but with increasing pH, they become ionized, which reduces the hydrophobic properties and consequently reduces the extraction efficiency. So pH 1 was considered as optimum. The effect of NaCl addition on the extraction efficiency was investigated. Extraction was performed from aqueous samples in the absence of NaCl and the presence of values of 0.1, 0.2, and 0.3 g of NaCl per 1 mL sample. The amount of extracted VMA increased by increasing the amount of NaCl in the aqueous sample. Its maximum was reached at 0.2 g and remained almost constant for values above this. Finally, the vortex time was examined. For this purpose, 3, 5, and 10 minutes were studied. It was observed that with increasing vortex time, the extraction yield increased, however, at 10 min, only a small amount improved over 5 min (about 5%). So 10 min was chosen as the optimal time. The best working conditions were as follows: NaCl amount, 0.2 g per 1 mL sample; sample volume, 1 mL; vortexing time, 10 min; sample pH, 1 and 1 mL of diethyl ether as an extraction solvent.
Optimization of CE separation

The choice of BGE type is crucial to achieving the satisfactory separation of the analyte of interest in CZE. Due to the acidic nature of the VMA, the best BGE is a buffer with pH > 4.11 (pH = pKa + 1 or more). Acetate (pH 4.75) and borate (pH 9.2) buffers enable partial or full ionization of the acidic analytes, respectively [49]. Due to the similar pKa values of the other catecholamine metabolites found in urine (e.g., homovanillic acid, 5-hydroxyindoleacetic acid, and 3-methoxytyramic acid) to VMA, the use of a slightly acidic acetate buffer allows them to be better separated since the analytes are only partially ionized [50]. So acetate BGE solution was selected for this work. However, since our main goal is to try to inject the extraction solvent directly into the CE, therefore it was necessary to change both the nature of the BGE and extraction solvent to allow direct injection. Consequently, in this study, CE separation was investigated for the analysis of VMA in the organic matrix. Injection of the organic solvents to CE with the fully aqueous BGE solution leads to serious deterioration of CE performance. Subsequently, ACN was picked as a suitable dilution medium [51]. So, a series of acetate /ACN (10-70% (v/v) were evaluated. BGE and diethyl ether incompatibilities were still observed for values less than 30% of ACN. But the successful separation was achieved for increased ACN concentrations. Besides, improved separation efficiency (up to 50% ACN) was observed. Also, an injection of diethyl ether diluted 1:1 (v/v) with methanol was performed to ensure enough compatibility of the injected sample with the BGE solution [50]. The effect of buffer concentration (20, 40, 60, 80, and 100 mmol·L⁻¹) as an effective factor on CE separation and resolution was explored and 60 mmol·L⁻¹ was selected as the optimum acetate buffer concentration. Final optimized BGE which consists of 60 mmol·L⁻¹ acetate buffer containing 50% (v/v) ACN (final apparent pH; pH after adding 50% (v/v) ACN is 5.73). Different voltage values ranging from +15 to +30 kV were investigated to select the optimized level. Higher separation voltage contributes good resolution, while low separation voltage leads to peak broadening. The voltage of +20 kV was chosen as optimal for acquiring the satisfactory efficiency of the separation and high resolution. In addition, the capillary temperature was fixed at 25 °C to obtain reproducible results. The effect of hydrodynamic injection time on sensitivity and separation efficiency was investigated at varying sampling times (2, 3, 4, 5, and 6 s at an injection pressure of 50 mbar). The results showed that applying a 3 s injection provided the most satisfactory signals. Broadened, overlapping peaks are observed for longer injection times.

Assay validation

The developed VALLE-CE-DAD method for the quantification of VMA in aqueous samples was validated according to the criteria suggested by the FDA, such as linear-
ity, the limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, specificity and ER [52].

**Linearity**

A calibration curve was acquired by plotting the peak area of the VMA against the nominal concentration of calibration standards analyzed in triplicate under the optimized VALLE-CE-DAD method using a least-squares linear regression model. The linearity of the method was checked by analyzing five calibration standard samples. The assay was found to be linear in the concentration range of 1.0 –14 µg·mL⁻¹. From the calibration curve, the linear equation was $y = 17.969x + 0.7211$ ($r^2 = 0.9986$), which demonstrated the excellent linearity of the method in the considered concentration range.

**Limits of detection and quantitation**

LOD and LOQ were determined based on a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ were found to be 0.30 and 1.0 µg·mL⁻¹, respectively, which indicates that the developed method is fit for the purpose. Normal concentrations of VMA in urine vary from 2.3 to 5.7 µg·mL⁻¹.

**Precision and accuracy**

The intra-day precisions were assessed for the developed VALLE-CE-DAD method by measuring five replicate injections at three different concentrations of a standard containing 1.0, 2.0 and 8.0 µg·mL⁻¹ of VMA. The inter-day precision was evaluated at the previously stated concentrations over three consecutive days. Precision was expressed as RSD%. The accuracy of the proposed method was evaluated by measuring the recovery of added known amount of VMA to a real urine sample (standard addition) via the implementation of five replicate extractions at three different concentrations of a sample containing 1.0, 2.0, and 8.0 µg·mL⁻¹ of VMA. Accuracy was expressed as RR and calculated by comparing the measured concentration according to the regression equation with added concentration (equation 1). Intra- and inter-day RSDs were below 4.30%, which indicates good repeatability of the proposed procedure. The RR was in the range of 87-109%, indicating that there is no significant matrix effect.

**Selectivity**

The selectivity of the developed method was tested by analyzing both the urine samples collected from volunteers and the spiked urine samples to check for interference from peaks at the migration time of the VMA. Figure 2 shows typical electropherograms for the analysis of VMA in real urine and spiked urine samples. It can be seen that the VMA can be determined without any interference from the other endogenous compounds found in urine. Other main peaks present in the blank urine sample may
be related to other acidic metabolites of catecholamines such as homovanillic acid, 3-methoxytyramic acid, and 5-hydroxy-indoleacetic acid, because they are acid, so they can be extracted into the extraction phase under the utilized optimal conditions of VALLE, and also their concentration in urine is much higher than that of the VMA [8]. Principally high concentration acidic compounds can be extracted and detected using here developed treatment method under optimized conditions.

**Figure 2.** Typical electropherograms of VMA extracted using developed VALLE-CE-DAD under optimized conditions (a) from a standard aqueous solution at concentration of 4 µg·mL⁻¹ (b) non-spiked urine sample and (c) urine sample with standard VMA spiked at concentration of 4 µg·mL⁻¹.

**The extraction recovery**

ER of VMA was calculated by comparing the peak area ratio after extraction from the standard at three concentration levels (1, 2, and 8 µg·mL⁻¹) with the peak area of direct injection of standards containing the same concentrations of VMA (equation 2). Under optimal conditions, the obtained ER for the VALLE method using diethyl ether were between 95-97%. The performed recovery experiments show the high efficiency of the used extraction procedure.
Peak identification and purity check

In order to determine the migration time of VMA, the entire developed method was used to extract VMA from a standard aqueous solution at a concentration of 4 µg·mL⁻¹ (figure 2a) and non-spiked urine sample as well (figure 2b). Figure 2c depicts the electropherogram of the spiked urine sample with standard VMA. Therefore, it is easy to conclude that a marked peak at migration time of 3.7 min is the peak of VMA. In addition, the purity of the peak was examined using the UV spectrum recorded by the DAD, which indicated that the peak was pure and that there was no co-eluted compound (figure 3). Figure 3a shows the selected peak (VMA) spectra. The signals overlap perfectly reaffirming the validity of the background correction. The similarity curve (figure 3b, solid trace) exhibits a profile very similar to and within the threshold curve limits (figure 3b, dotted trace) and, the peak purity ratio (diamond points) is clear within the green band. The purity factor (999.95) is within the calculated threshold limit (998.03).

Figure 3. Peak purity evaluation by ChemStation® software. (a) The UV spectra of selected peak at migration time of 3.7 min corresponds to VMA recorded at different wavelengths and (b) the similarity curve (solid trace), threshold curve (dotted trace), and peak purity ratio (diamond points); the placement of the similarity curve below the threshold curve indicates the purity of the peak, which is confirmed by the placement of the peak purity ratio within the green zone and below the red zone.

Real sample analysis

The proposed VALLE-CE-DAD method was applied to determine the VMA in the urine samples from three healthy volunteers. Electropherograms of the urine samples are shown in figure 4. The content of VMA in the tested urine samples was found to be below the LOQ of the method.
VMA is often measured for the diagnosis of neuroblastoma and pheochromocytoma. Commonly VMA is measured using HPLC or GC-MS platforms. These assays have several analytical performance advantages including improved selectivity and sensitivity [20]. However, such procedures are expensive and complicated. SPE [53, 54], dispersive liquid-liquid microextraction [24], microextraction by packed sorbent [17] and dilute-and-shoot approaches [22, 23, 55, 56] have been described for sample clean-

Figure 4. The electropherograms of three urinary sample solutions from healthy volunteers based on VALLE-CE-DAD method under optimized conditions.
up prior to chromatographic analyses. CE has also been used for quantitation of VMA [8, 36, 38-41], because of its rapid separation, high resolution, small injection volume, and low cost. Sample treatment increases cost, turnaround time, method complexity, and in some cases it may hinder automation. Moreover, sample treatment contributes to method robustness [20]. Prior to CE analyses, simple and fast treatment methods such as centrifugation, dilution and filtration have been used, but these methods suffer from weak selectivity in real samples [8, 36, 39, 40]. Recently, VALLE due to its adequate clean-up used to treat urine samples prior to CE analysis [41]. However, the evaporation and reconstitution steps are time-consuming and complicate the process. In addition, these steps can preclude automation and are also a source of error [8]. We developed a simple and rapid method for quantitative analysis of urinary VMA. Our developed VALLE approach eliminates evaporation and reconstitution and minimizes sample processing. Direct injection of extraction solvent to CE enabled the straightforward analysis of VMA, which represents a distinct advantage over current methods. Compared to previous studies [38, 41] that used the VALLE method (Table 1), the precision of our method has improved (RSD% ≤ 4.3) and the sample clean-up time has also been reduced (15 min). In the developed method, in the absence of the internal standard, RSDs of less than 4.3% have been obtained, which indicates the reliability of the method. The proposed method can be easily automated.

Conclusion

CE with features such as higher separation efficiency and faster separation can be a good option for biomedical applications and clinical analyses. The direct injection of organic solvent into CE can be achieved using a binary solvent running buffer. The CE-DAD method for the quantitative analysis of VMA in human urine is described using a simple, rapid, and straightforward VALLE pretreatment. Diethyl ether was used as the extraction solvent for efficient sample clean-up. Direct injection of the diethyl ether + methanol reduces the sample preparation time. Moreover, by eliminating the error prone evaporation and reconstitution steps, the reliability of the method is improved. Besides, under these conditions, there is no need to use the internal standard.

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A capillary electrophoresis procedure for quantification of vanillylmandelic acid

Disclosure statement

The authors declare that they have no conflict of interest.

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