

# Analytical Methods

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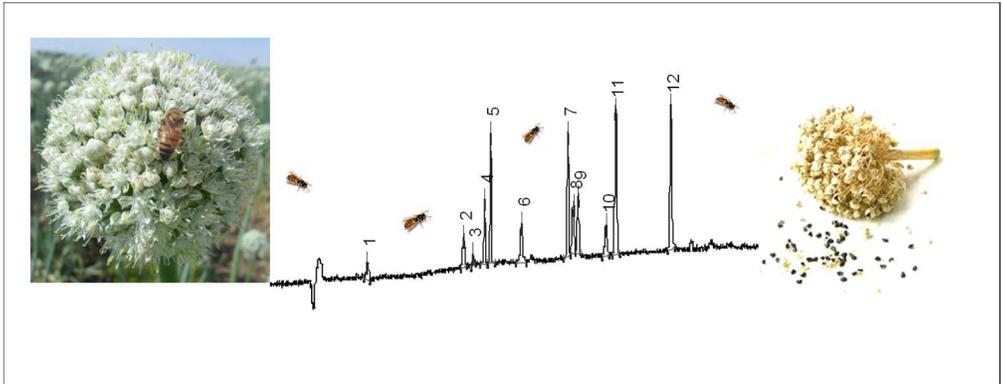
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3 **ANALYSIS OF PHENOLIC COMPOUNDS IN ONION NECTAR BY MINIATURIZED OFF-LINE**  
4 **SOLID PHASE EXTRACTION-CAPILLARY ZONE ELECTROPHORESIS**  
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**Abstract**

Onion is an important vegetable crop which depends heavily on cross-pollinating insects for any significant increase in seed production. Nectar is the most important floral reward offered by plants. Minor components such as phenolic compounds in nectar affect bee foraging. A simultaneous determination of 4-vinylphenol, catechin, naringenin, rutin, cinnamic acid, syringic acid, chlorogenic acid, apigenin, vanillic acid, luteolin, quercetin, and caffeic acid in onion nectar lines by CZE-UV is reported. Limited nectar amounts make it difficult to apply most commonly employed methods of extraction for phenolic compounds. Thus, the extraction of phenolic compounds in nectar was performed by a solid phase extraction (SPE) using a home-made minicolumn packed with suitable filtering material ( $C_{18}$ , 50 mg) connected to a vacuum pump. Effects of several important factors affecting extraction efficiency, as well as electrophoretic performance were investigated to acquire the optimum conditions. Under the purposed conditions, the analytes could be separated within 8 min in a 50 cm effective length capillary (75  $\mu\text{m}$  ID) at a separation voltage of 25 kV in boric acid 30  $\text{mmol L}^{-1}$  as BGE (pH 9.5). Sample results suggest that phenolic composition have a great influence on bee preference. Furthermore, the markedly qualitative and quantitative analytical differences in the phenolic profile of onion lines found in this study contribute to the understanding of the factors that affect onion pollination for hybrid seed production.

**Key words:** capillary zone electrophoresis (CZE), Solid-phase extraction (SPE), phenolic compounds, onion nectar.

## 1. Introduction

Phenolic compounds are one of the most important groups of compounds occurring in plants, where they are widely distributed, comprising at least 8000 different known structures [1].

Onion (*Allium cepa* L.) is an important vegetable crop which depends heavily on cross-pollinating insects for any significant increase in seed production. Cross pollination is the most frequent way for seed production and insects are necessary for pollen transfer. Honeybees have been reported as the most efficient and practical pollinator of this crop because of their affinity for pollen and nectar and efficiency at pollen transfer [2]. It is well known that onion is one of the richest sources of dietary flavonoids in many countries [3-5].

Of the diverse floral rewards offered by plants, nectar is the most important. It is composed of sugars which constitute the major energy source for pollinators, and may contain a variety of other solute compounds, e.g. phenols, amino acids, reducing acids, lipids, proteins, and alkaloids [6]. Minor components of nectar might directly affect bees foraging, therefore pollination further accentuate the necessity for chemical data on nectar. Nectar usually does not repel bees, but a particular nectar may be less attractive than nectar of competing flowers [7]. Honeybees have distaste for flowers of certain varieties of onion and avoid visiting them [8].

Although the presence of secondary compounds in floral nectar may provide reproductive benefits to plants in some special cases, it is predicted to have detrimental effects if pollinators are deterred from visiting flowers. Paradoxically, floral nectar of some plant species also contains secondary compounds such as phenols and alkaloids that occur in leaves, stems, and roots to defend against attack by herbivores and microorganisms [9]. Phenolic substances are quite widespread in nectars. Their accumulation may make the nectar toxic, so that it then becomes repellent to some visitors [10].

Although phenolic compounds have been widely studied in honey [11], there is a lack of knowledge about the profiles of phenolic substances in onion nectar samples. Compounds such as quercetin, kaempferol, luteolin, naringenin, cinnamic, vanillic, or caffeic acid have been reported as the most important phenols found in honey, pollen, propolis and other bee products related to floral nectar [9, 10]. Nectar has been analyzed in acacia by HPLC-DAD [12], rosemary by HPLC-MS [13] with the objective to find biochemical markers. To our knowledge, nectar from onion flowers has not been previously analyzed by CE or HPLC methods.

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3 For the determination of individual compounds, although many traditional sample-preparation  
4 methods for flavonoids are still in use, there have been trends in recent years towards: (1) use  
5 of smaller initial sample sizes, small volumes or no organic solvents; (2) greater specificity or  
6 greater selectivity in extraction; (3) higher recoveries or better reproducibility; and, (4) increased  
7 potential for automation. Thus, sample treatment by means of pressurized liquid extraction  
8 (PLE), microwave-assisted extraction (MAE), supercritical-fluid extraction (SFE), solid-phase  
9 extraction (SPE), matrix solid-phase dispersion (MSPD) and solid-phase micro-extraction  
10 (SPME) deserve more attention [14-16].

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17 On the other hand, CE is becoming increasingly recognized as an important analytical  
18 separation technique for the separation and quantification of phenolic compounds due to its  
19 speed, efficiency, reproducibility, ultra small sample volume requirements, low cost and facility  
20 for clearing the contaminants. At the moment, despite of the advantages on the application of  
21 CE, there are no reports dealing with the determination of phenolic compounds in onion nectar  
22 by this technique.

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29 The aim of our work was to develop a sensitive and reliable method for the extraction,  
30 separation and quantitation of representative phenolic compounds in onion nectar (4-  
31 vinylphenol, catechin, naringenin, rutin, cinnamic acid, syringic acid, chlorogenic acid, apigenin,  
32 vanillic acid, luteolin, quercetin, and caffeic acid) by off-line SPE-CZE-UV.

## 33 34 35 36 37 **2. Materials and methods**

### 38 39 *2.1. Plant Materials.*

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41 An open pollinated (OP) onion cultivar, Valcatorce from INTA, as well as three male sterile lines  
42 (MSLs), MSL 1, MSL 2, and MSL 3 from Enza Zaden were cultivated in a randomized complete  
43 block design with three replicates for each cultivar under a cage (4 by 15 m) to isolate the  
44 materials from other pollinators, at the Institute of Horticulture (Agronomy Faculty, UNCuyo,  
45 Mendoza, Argentina). No pesticides were used along the experiment. The plants flowered from  
46 November to December, 2012. Onion nectar was obtained in blossom.

### 47 48 49 *2.2. Chemical and Reagents*

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Ultrapure water (resistivity 18.3 MΩcm) obtained from Barnstead EASY pure RF water system  
(Iowa, USA) was used to prepare solutions including the background electrolyte. Catechin,

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3 naringenin, rutin, cinnamic acid, syringic acid, chlorogenic acid, apigenin, vanillic acid,  
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5 quercetin, luteolin, and caffeic acid were purchased from Sigma (St. Louis, MO).

### 6 7 *2.3. Solutions and samples*

#### 8 9 *2.3.1. Standard solutions*

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11 Standard stock solutions of the analytes were prepared by dissolving an appropriate amount of  
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13 each pure substance in 10 mL of a HPLC-grade methanol to obtain a final concentration of  
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15 1000 mg L<sup>-1</sup>. The resulting solutions were stored at 4 °C in amber glasses.

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17 Working standard solutions at a 5 mg L<sup>-1</sup> concentration were prepared on a daily basis by  
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19 diluting appropriate aliquots of the previous standard stock solutions in methanol. Before use, all  
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21 solutions were filtered through 0.22 µm nylon filters.

#### 22 23 *2.3.2. Background electrolyte*

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25 Phenolic compounds were separated in a background electrolyte (BGE) consisting of a 30  
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27 mmol L<sup>-1</sup> boric acid solution, pH 9.5 that was prepared by weighing the required amount of  
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29 boric acid and adjusting its pH with sodium hydroxide.

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31 All solutions and buffers were degassed by sonication for 5 min before. pH measurements were  
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33 made with an Altronix model TPX-I pH meter furnished with a combined glass electrode.

#### 34 35 *2.3.3. Nectar samples and SPE procedure*

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37 In order to obtain the nectar on the most natural way, and preserve it in similar conditions as it is  
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39 in the plant, we found that the most effective way of extraction was to separate freshly opened  
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41 flowers from umbels, removing anthers, filaments and peduncle, and immediately centrifuging  
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43 (13000 rpm, 30 min, 4°C) into a 1,5 mL microtube. It was possible to extract around 10 µL of  
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45 nectar from each umbel. Then, nectar was stored at -80 °C until analyses.

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47 A SPE procedure was developed and optimised to isolate the phenolic fraction and to  
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49 preconcentrate the nectar samples. The extraction of phenolic compounds in nectar was  
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51 performed by a solid phase extraction (SPE) using a home-made column packed with suitable  
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53 filtering material. C<sub>18</sub> cartridges (50 mg) were made in 1 mL syringes using 25 mg of glass wool  
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55 as frits. These cartridges were placed in a vacuum elution apparatus (Varian Vac Elut 20  
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57 manifold and a Vacubrand vacuum pump ME 2C) and preconditioned passing 5 mL of  
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59 methanol and 5 mL of acid water (water pH 2, HCl). The nectar samples (50 µg) were  
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thoroughly mixed with five parts (1:5, w/v) of acidic water until completely homogenization and

carefully loaded onto the pre-conditioned column, leaving the sample on the solid phase under vacuum. Then, the column was washed with 1 mL of acid water (water pH 2, with HCl). The phenolic compounds present in nectar remained in the column while sugars and other highly polar compounds were eluted with the aqueous solvent. The whole phenolic fraction was eluted with methanol (500  $\mu$ L). The eluent was directly injected and analyzed by CZE.

#### 2.4. Capillary zone electrophoresis (CZE)

CZE separations were carried out using a Capel<sup>TM</sup> 105M apparatus equipped with a 57 cm full length, 50 cm effective length, 75  $\mu$ m ID and 375  $\mu$ m OD fused silica capillary. The capillary tube was conditioned prior to its daily use by flushing with water (5 min), 0.1 mol L<sup>-1</sup> NaOH for 5 min, followed by water for another 2 min and finally with the buffer for 5 min. The running buffer was boric acid 30 mmol L<sup>-1</sup> pH 9.5. The separation voltage was 25 kV and the capillary temperature was 25 °C. Samples were injected by hydrodynamic injection at 30 mbar for 2 s. Electropherograms were recorded at 290 nm. Between runs, the capillary was flushed with water (2 min), 0.1 mol L<sup>-1</sup> NaOH (2 min), water (2 min) and fresh buffer (2 min). The capillary tube was rinsed with 0.1 mol L<sup>-1</sup> NaOH for 10 min, then with water for 10 min every day after use.

#### 2.5. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA), and means were compared using Tukey test. All the analyses were done in triplicate. The results were significant at  $P < 0.05$  unless specified otherwise. Statistical analyses were carried out using statistical package STATISTICA 7.0 for Windows (from StatSoft, Tulsa, OK).

### 3. Results and discussion

#### 3.1. Optimization of extraction conditions

The extraction step is necessary due to matrix effects taking into account that nectar is a salty-aqueous matrix composed mainly by sugars. Several variables were tested to determine the most suitable conditions for the analysis of phenolic compounds in onion nectar. The yield and repeatability of the extraction was affected by factors such as the type of stationary phase of the cartridge, cartridge conditioning, sample pH and volume, elution plug volume and composition. In order to determine the optimal extraction conditions, the univariate approach was performed.

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3 In this step of method development, the real samples of nectar were investigated. The variation  
4 coefficients (CV) for optimization of extraction conditions were calculated as relative standard  
5 deviations of corrected area (peak area/tr) for the triplicate analyses of real samples.  
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### 8 9 *3.1.1. Effect of the type of cartridge*

10 Limited nectar amounts make it difficult to apply most commonly employed methods of  
11 extraction for phenolic compounds. Thus, in order to accurately handle the ultra small sample  
12 amounts, a homemade minicolumn was prepared by filling an empty syringe (1 mL) with 50 mg  
13 of the material. To avoid loss of filling material when the sample solution passes through the  
14 minicolumn, glass wool (25 mg) was placed at both sides of the column. The minicolumn was  
15 then connected to a vacuum pump.  
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18 Sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids,  
19 which would reduce their retention [14]. In this study, seven different SPE cartridges were  
20 compared using the method described above, and parameters affecting the recoveries of  
21 phenolic compounds, including the volume and composition of the wash buffer, and the volume  
22 of the elution solvent were optimized. Three kind of bonded silica sorbents: C<sub>8</sub> (particle size: 56  
23 μm), C<sub>18</sub> (55 μm), and Strata-X (28-34 μm); two types of ambertite resin: XAD 1180 (0.03 μm)  
24 and XAD 16 (0.01 μm), and two kinds of nanostructured materials: Al<sub>2</sub>O<sub>3</sub> phase X (crystallite  
25 size: 0.13 μm), SiO<sub>2</sub> (nano acetic) were tested. Figure 1 shows the responses obtained for the  
26 seven SPE cartridges tested. From the figure it is clear that C<sub>18</sub> sorbent was the most efficient.  
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### 38 39 *3.1.2. Effect of conditioning, rinse and elution solvent volume on the SPE extraction*

40 An important parameter in SPE is the breakthrough volume (initial analyte volume) because  
41 enrichment factor depends on the volumes of analyte and volume of eluate. In other words, the  
42 breakthrough volume is the maximum solution volume in which the analytes can be retained  
43 within the acceptable recovery range [17]. As volume of nectar is limited, a sample of 50 μL was  
44 set as the volume to use on the SPE extraction. The amount of sample was reduced to 50 μg  
45 compared with 0.1 g of nectar or 10-100 g used in other studies purposed for honey samples  
46 [18-21]. Remaining this volume constant, the other factors of SPE were tested.  
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53 For column conditioning, different solvent systems were tested: methanol and water and  
54 methanol and acid water (pH 2 with HCl) between 5 to 10 mL of each one. The best results  
55 were obtained with 5 mL of methanol followed by 5 mL of acid water.  
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3 After sample loading, two wash buffers, i.e. water and acid water (pH 2 with HCl) were  
4 investigated. Different volumes of rinse were tested, between 250  $\mu\text{L}$  and 5 mL. It was observed  
5 that 1 mL acid water is sufficient  
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8 Then, 250  $\mu\text{L}$  - 2 mL of methanol and acetonitrile were tested for the elution procedure, it was  
9 observed that 500  $\mu\text{L}$  were adequate to obtain the maximum recoveries.  
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11 Taken together, the conditioning, rinse and elution conditions that provided the highest recovery  
12 values were the following: 1) 5 mL methanol, 2) 5 mL acid water (pH 2), 3) sample loading (50  
13  $\mu\text{g}$  nectar mixed with five parts (1:5, w/v) of acidic water, 4) 1 mL acid water, 5) 500  $\mu\text{L}$   
14 methanol.  
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### 19 3.2. Optimization of CE instrumental parameters

20 With a view to establishing the best possible compromise between sensitivity, resolution and  
21 analysis time in the separation of all analytes, the following parameters were consecutively  
22 optimized: BGE composition and concentration, injection volume and mode, and other  
23 electrophoretic parameters such as electrophoretic separation voltage, and capillary  
24 temperature and conditioning.  
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30 The buffer pH plays an important role for improving selectivity in CE especially for closely  
31 related compounds, because it affects both the overall charges of the solute and the  
32 electroosmotic flow (EOF). Taking into consideration the acidic nature such of the analytes  
33 under study the effect of the buffer pH was investigated within the range of 8.0/10.0 at a fixed  
34 buffer concentration, adjusted by 0.1 mol L<sup>-1</sup> NaOH and 0.1 mol L<sup>-1</sup> HCl. It was found that  
35 when the pH was lower than 9.0, the resolution was poor. At pH 9.5 baseline separation was  
36 achieved.  
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43 Sodium tetraborate and boric acid were tested as BGEs, but the one producing the best results  
44 considering selectivity, reproducibility, baseline and current performance, was boric acid.  
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46 Keeping other parameters constant (pH: 9.5, 25 kV, 25 °C) the buffer concentration was varied  
47 from 10 to 75 mM. Increases in migration times as well as current were observed when the  
48 concentration of buffer increased. Resolution also increased for higher buffer concentrations,  
49 but no appreciable improvements were observed for buffer concentrations above 30 mM. So,  
50 baseline separation was obtained with 30 mmol L<sup>-1</sup> boric acid buffer, pH 9.5.  
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3 The separation voltage affects the electric field strength, which in turn affects the EOF and the  
4 migration velocity of charged particles, which determine the migration time of the analytes. The  
5 effect of the applied voltage was studied over the range 5-35 kV. A potential of 25 kV was found  
6 to provide the best results in terms of run time and resolution between peaks.  
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10 The effect of temperature on electrophoretic separation was examined over the range 15-30 °C.  
11 A temperature of 25 °C was selected as optimal because it provided the best compromise  
12 between migration time (MT) and peak resolution (RS). In fact, raising the capillary temperature  
13 reduced migration times through a decreased electrolyte viscosity, but also led to lower RS  
14 values.  
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18 The injection mode giving the best response concerning reproducibility and linear range was the  
19 hydrodynamic mode. Injection parameters were optimized by varying the lengths of sample (2-8  
20 s) and pressure injection until optimal conditions were reached. The best results were obtained  
21 for the following experimental parameters: hydrodynamic injection mode 30 mbar, 2 s.  
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### 24 *3.3. Repeatability, reproducibility and detection limits*

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26 The electropherograms were monitored at 290 nm. A typical electropherogram for the standard  
27 mixture solution under the optimum conditions is shown in Fig. 2. Baseline separation for all  
28 analytes can be achieved within 8 min.  
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32 In order to determine the repeatability of the methodology, replicate injections ( $n = 6$ ) of a  
33 standard mixture solution ( $2.00 \mu\text{g mL}^{-1}$  for each analyte) under the selected optimum conditions  
34 were carried out. The intraday % RSDs of the migration time, corrected area (peak area/tr),  
35 were between 0.8 and 1.6, 1.7 and 3.8, respectively. The interday values for the same  
36 performance criteria were 0.8–1.8 and 1.6–4.9, respectively.  
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40 Calibration curves for the determination of the 12 compounds were constructed under the  
41 optimum conditions. Six concentrations were used for each calibration curve. As shown in Table  
42 1, most of the correlation coefficients of area ratio equations were  $>0.96$ . The corrected peak  
43 area and concentration of each analyte were subjected to regression analysis to obtain the  
44 calibration equations and correlation coefficients, and the results showed that within the  
45 concentration range there was an excellent correlation between the corrected peak area and  
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3 the concentration of each analyte. The limits of detection (LODs) and quantification (LOQs)  
4 were evaluated on the basis of signal-to-noise ratios (S/N) of 3 and 10, respectively (Table 1).  
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7 In order to determine the accuracy of this method, a 500  $\mu\text{g}$  of nectar (from all the onion lines  
8 tested) were collected and divided into 10 portions of 50  $\mu\text{g}$  each. The proposed method was  
9 applied to six portions and the average concentrations determined for each compound were  
10 taken as a base value. Then, known quantities of the analytes were added to the other aliquots,  
11 and the phenolic compounds determined following the recommended procedure. The recovery  
12 studies showed satisfactory robustness leading recoveries higher than 82.00% and lower than  
13 110.00% for all the analytes under study.  
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#### 20 21 *3.4. Phenolic composition of onion lines*

22 The optimized CZE method was then applied to determine phenolic compounds in nectar of  
23 different onion lines. Representative electropherograms are shown in Fig. 3. Markedly  
24 qualitative and quantitative analytical differences in the phenolic profile of onion lines were  
25 observed.  
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30 The results of the quantitative determination of phenolic acids and flavonoids are presented in  
31 Table 2. Of the 12 compounds tested, only 9 were found in the samples. The total amount of  
32 phenolic compounds ranged between 6.52 and 11.78  $\text{mg L}^{-1}$ . One of the male sterile lines (MSL  
33 1) was the nectar with highest phenolic content. Naringenin was found only in male sterile lines  
34 while the open pollinated (OP) line showed the highest amounts of luteolin.  
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39 Phenolics have been reported as insect deterrents or repellents [22-24]. Most of the phenolic  
40 compounds found in our study are in agreement with those reported in honey; these could be  
41 explained due to their solubility in aqueous solutions [25]. In a previous work [26], we have  
42 demonstrated that nectar chemical composition has a great influence on bee behavior and seed  
43 production. Significant differences on chemical composition as well as morphological aspects  
44 were found between open pollinated and male sterile lines; being male sterile lines the less  
45 pollinated. From the results shown in Table 2, it can be assumed that compounds such as  
46 naringenin and luteolin may influence bee foraging behavior, as deterrent and attracting agents,  
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#### 4. Conclusions

At the moment, despite its advantages, to our knowledge there are no reports on the application of SPE-CE-UV for the determination of phenolic compounds in onion nectar. A simplified extraction and rapid CZE method was developed for the isolation and separation of the main phenolic compounds present in onion nectar. Solid phase extraction is used to isolate phenolic compounds from nectar and baseline resolution is achieved in less than 8 min using boric acid buffer system which enabled accurate and reproducible quantification of phenolic compounds in onion nectar. The short analysis time coupled with greatly reduced solvent consumption made it a viable alternative to traditional HPLC. The amount of sample requirement was reduced being up to 2000 times smaller than traditional methods. It has to be pointed out that further studies could greatly benefit from other detection systems such as laser-induced fluorescence and mass spectrometry in order to achieve lower detection limits and identify new phenols in onion nectar.

The markedly qualitative and quantitative analytical differences in the phenolic profile of male sterile and open pollinated lines found in this study contribute to the understanding of the factors that affect onion pollination for hybrid seed production.

#### Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2189), University of Cuyo (6/A445), and National Vegetable Flower and Aromatics Program of INTA. We thank Enza Zaden Seed Company for providing plant material and Héctor Fuligna and Gustavo Lascalea for their valuable support.

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3 ANALYSIS OF PHENOLIC COMPOUNDS IN ONION NECTAR BY MINIATURIZED OFF-LINE  
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5 SOLID PHASE EXTRACTION-CAPILLARY ZONE ELECTROPHORESIS  
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**Abstract**

Onion is an important vegetable crop which depends heavily on cross-pollinating insects for any significant increase in seed production. Nectar is the most important floral reward offered by plants. Minor components such as phenolic compounds in nectar affect bee foraging. A simultaneous determination of 4-vinylphenol, catechin, naringenin, rutin, cinnamic acid, syringic acid, chlorogenic acid, apigenin, vanillic acid, luteolin, quercetin, and caffeic acid in onion nectar lines by CZE-UV is reported. Limited nectar amounts make it difficult to apply most commonly employed methods of extraction for phenolic compounds. Thus, the extraction of phenolic compounds in nectar was performed by a solid phase extraction (SPE) using a home-made minicolumn packed with suitable filtering material (C<sub>18</sub>, 50 mg) connected to a vacuum pump. Effects of several important factors affecting extraction efficiency, as well as electrophoretic performance were investigated to acquire the optimum conditions. Under the purposed conditions, the analytes could be separated within 8 min in a 50 cm effective length capillary (75 µm ID) at a separation voltage of 25 kV in boric acid 30 mmol L<sup>-1</sup> as BGE (pH 9.5). Sample results suggest that phenolic composition have a great influence on bee preference. Furthermore, the markedly qualitative and quantitative analytical differences in the phenolic profile of onion lines found in this study contribute to the understanding of the factors that affect onion pollination for hybrid seed production.

**Key words:** capillary zone electrophoresis (CZE), Solid-phase extraction (SPE), phenolic compounds, onion nectar.

## 1. Introduction

Phenolic compounds are one of the most important groups of compounds occurring in plants, where they are widely distributed, comprising at least 8000 different known structures [1].

Onion (*Allium cepa* L.) is an important vegetable crop which depends heavily on cross-pollinating insects for any significant increase in seed production. Cross pollination is the most frequent way for seed production and insects are necessary for pollen transfer. Honeybees have been reported as the most efficient and practical pollinator of this crop because of their affinity for pollen and nectar and efficiency at pollen transfer [2]. It is well known that onion is one of the richest sources of dietary flavonoids in many countries [3-5].

Of the diverse floral rewards offered by plants, nectar is the most important. It is composed of sugars which constitute the major energy source for pollinators, and may contain a variety of other solute compounds, e.g. phenols, amino acids, reducing acids, lipids, proteins, and alkaloids [6]. Minor components of nectar might directly affect bees foraging, therefore pollination further accentuate the necessity for chemical data on nectar. Nectar usually does not repel bees, but a particular nectar may be less attractive than nectar of competing flowers [7]. Honeybees have distaste for flowers of certain varieties of onion and avoid visiting them [8].

Although the presence of secondary compounds in floral nectar may provide reproductive benefits to plants in some special cases, it is predicted to have detrimental effects if pollinators are deterred from visiting flowers. Paradoxically, floral nectar of some plant species also contains secondary compounds such as phenols and alkaloids that occur in leaves, stems, and roots to defend against attack by herbivores and microorganisms [9]. Phenolic substances are quite widespread in nectars. Their accumulation may make the nectar toxic, so that it then becomes repellent to some visitors [10].

Although phenolic compounds have been widely studied in honey [11], there is a lack of knowledge about the profiles of phenolic substances in onion nectar samples. Compounds such as quercetin, kaempferol, luteolin, naringenin, cinnamic, vanillic, or caffeic acid have been reported as the most important phenols found in honey, pollen, propolis and other bee products related to floral nectar [9, 10]. Nectar has been analyzed in acacia by HPLC-DAD [12], rosemary by HPLC-MS [13] with the objective to find biochemical markers. To our knowledge, nectar from onion flowers has not been previously analyzed by CE or HPLC methods.

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3 For the determination of individual compounds, although many traditional sample-preparation  
4 methods for flavonoids are still in use, there have been trends in recent years towards: (1) use  
5 of smaller initial sample sizes, small volumes or no organic solvents; (2) greater specificity or  
6 greater selectivity in extraction; (3) higher recoveries or better reproducibility; and, (4) increased  
7 potential for automation. Thus, sample treatment by means of pressurized liquid extraction  
8 (PLE), microwave-assisted extraction (MAE), supercritical-fluid extraction (SFE), solid-phase  
9 extraction (SPE), matrix solid-phase dispersion (MSPD) and solid-phase micro-extraction  
10 (SPME) deserve more attention [14-16].

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19 On the other hand, CE is becoming increasingly recognized as an important analytical  
20 separation technique for the separation and quantification of phenolic compounds due to its  
21 speed, efficiency, reproducibility, ultra small sample volume requirements, low cost and facility  
22 for clearing the contaminants. At the moment, despite of the advantages on the application of  
23 CE, there are no reports dealing with the determination of phenolic compounds in onion nectar  
24 by this technique.

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The aim of our work was to develop a sensitive and reliable method for the extraction,  
separation and quantitation of representative phenolic compounds in onion nectar (4-  
vinylphenol, catechin, naringenin, rutin, cinnamic acid, syringic acid, chlorogenic acid, apigenin,  
vanillic acid, luteolin, quercetin, and caffeic acid) by off-line SPE-CZE-UV.

## 2. Materials and methods

### 2.1. Plant Materials.

An open pollinated (OP) onion cultivar, Valcatorce from INTA, as well as three male sterile lines (MSLs), MSL 1, MSL 2, and MSL 3 from Enza Zaden were cultivated in a randomized complete block design with three replicates for each cultivar under a cage (4 by 15 m) to isolate the materials from other pollinators, at the Institute of Horticulture (Agronomy Faculty, UNCuyo, Mendoza, Argentina). No pesticides were used along the experiment. The plants flowered from November to December, 2012. Onion nectar was obtained in blossom.

### 2.2. Chemical and Reagents

Ultrapure water (resistivity 18.3 M $\Omega$ cm) obtained from Barnstead EASY pure RF water system (Iowa, USA) was used to prepare solutions including the background electrolyte. Catechin,

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3 naringenin, rutin, cinnamic acid, syringic acid, chlorogenic acid, apigenin, vanillic acid,  
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5 quercetin, luteolin, and caffeic acid were purchased from Sigma (St. Louis, MO).  
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### 7 *2.3. Solutions and samples*

#### 8 *2.3.1. Standard solutions*

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10 Standard stock solutions of the analytes were prepared by dissolving an appropriate amount of  
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12 each pure substance in 10 mL of a HPLC-grade methanol to obtain a final concentration of  
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14 1000 mg L<sup>-1</sup>. The resulting solutions were stored at 4 °C in amber glasses.  
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17 Working standard solutions at a 5 mg L<sup>-1</sup> concentration were prepared on a daily basis by  
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19 diluting appropriate aliquots of the previous standard stock solutions in methanol. Before use, all  
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21 solutions were filtered through 0.22 µm nylon filters.  
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#### 23 *2.3.2. Background electrolyte*

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25 Phenolic compounds were separated in a background electrolyte (BGE) consisting of a 30  
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27 mmol L<sup>-1</sup> boric acid solution, pH 9.5 that was prepared by weighing the required amount of  
28  
29 boric acid and adjusting its pH with sodium hydroxide.  
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31 All solutions and buffers were degassed by sonication for 5 min before. pH measurements were  
32  
33 made with an Altronix model TPX-I pH meter furnished with a combined glass electrode.  
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#### 35 *2.3.3. Nectar samples and SPE procedure*

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37 In order to obtain the nectar on the most natural way, and preserve it in similar conditions as it is  
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39 in the plant, we found that the most effective way of extraction was to separate freshly opened  
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41 flowers from umbels, removing anthers, filaments and peduncle, and immediately centrifuging  
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43 (13000 rpm, 30 min, 4°C) into a 1,5 mL microtube. It was possible to extract around 10 µL of  
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45 nectar from each umbel. Then, nectar was stored at -80 °C until analyses.  
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48 A SPE procedure was developed and optimised to isolate the phenolic fraction and to  
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50 preconcentrate the nectar samples. The extraction of phenolic compounds in nectar was  
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52 performed by a solid phase extraction (SPE) using a home-made column packed with suitable  
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54 filtering material. C<sub>18</sub> cartridges (50 mg) were made in 1 mL syringes using 25 mg of glass wool  
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56 as frits. These cartridges were placed in a vacuum elution apparatus (Varian Vac Elut 20  
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58 manifold and a Vacuubrand vacuum pump ME 2C) and preconditioned passing 5 mL of  
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60 methanol and 5 mL of acid water (water pH 2, HCl). The nectar samples (50 µg) were  
thoroughly mixed with five parts (1:5, w/v) of acidic water until completely homogenization and

carefully loaded onto the pre-conditioned column, leaving the sample on the solid phase under vacuum. Then, the column was washed with 1 mL of acid water (water pH 2, with HCl). The phenolic compounds present in nectar remained in the column while sugars and other highly polar compounds were eluted with the aqueous solvent. The whole phenolic fraction was eluted with methanol (500  $\mu$ L). The eluent was directly injected and analyzed by CZE.

#### 2.4. Capillary zone electrophoresis (CZE)

CZE separations were carried out using a Capel<sup>TM</sup> 105M apparatus equipped with a 57 cm full length, 50 cm effective length, 75  $\mu$ m ID and 375  $\mu$ m OD fused silica capillary. The capillary tube was conditioned prior to its daily use by flushing with water (5 min), 0.1 mol L<sup>-1</sup> NaOH for 5 min, followed by water for another 2 min and finally with the buffer for 5 min. The running buffer was boric acid 30 mmol L<sup>-1</sup> pH 9.5. The separation voltage was 25 kV and the capillary temperature was 25 °C. Samples were injected by hydrodynamic injection at 30 mbar for 2 s. Electropherograms were recorded at 290 nm. Between runs, the capillary was flushed with water (2 min), 0.1 mol L<sup>-1</sup> NaOH (2 min), water (2 min) and fresh buffer (2 min). The capillary tube was rinsed with 0.1 mol L<sup>-1</sup> NaOH for 10 min, then with water for 10 min every day after use.

#### 2.5. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA), and means were compared using Tukey test. All the analyses were done in triplicate. The results were significant at  $P < 0.05$  unless specified otherwise. Statistical analyses were carried out using statistical package STATISTICA 7.0 for Windows (from StatSoft, Tulsa, OK).

### 3. Results and discussion

#### 3.1. Optimization of extraction conditions

The extraction step is necessary due to matrix effects taking into account that nectar is a salty-aqueous matrix composed mainly by sugars. Several variables were tested to determine the most suitable conditions for the analysis of phenolic compounds in onion nectar. The yield and repeatability of the extraction was affected by factors such as the type of stationary phase of the cartridge, cartridge conditioning, sample pH and volume, elution plug volume and composition. In order to determine the optimal extraction conditions, the univariate approach was performed.

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3 In this step of method development, the real samples of nectar were investigated. The variation  
4 coefficients (CV) for optimization of extraction conditions were calculated as relative standard  
5 deviations of corrected area (peak area/tr) for the triplicate analyses of real samples.  
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### 9 10 3.1.1. *Effect of the type of cartridge*

11 Limited nectar amounts make it difficult to apply most commonly employed methods of  
12 extraction for phenolic compounds. Thus, in order to accurately handle the ultra small sample  
13 amounts, a homemade minicolumn was prepared by filling an empty syringe (1 mL) with 50 mg  
14 of the material. To avoid loss of filling material when the sample solution passes through the  
15 minicolumn, glass wool (25 mg) was placed at both sides of the column. The minicolumn was  
16 then connected to a vacuum pump.  
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23 Sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids,  
24 which would reduce their retention [14]. In this study, seven different SPE cartridges were  
25 compared using the method described above, and parameters affecting the recoveries of  
26 phenolic compounds, including the volume and composition of the wash buffer, and the volume  
27 of the elution solvent were optimized. Three kind of bonded silica sorbents: C<sub>8</sub> (particle size: 56  
28 μm), C<sub>18</sub> (55 μm), and Strata-X (28-34 μm); two types of ambertite resin: XAD 1180 (0.03 μm)  
29 and XAD 16 (0.01 μm), and two kinds of nanostructured materials: Al<sub>2</sub>O<sub>3</sub> phase X (crystallite  
30 size: 0.13 μm), SiO<sub>2</sub> (nano acetic) were tested. Figure 1 shows the responses obtained for the  
31 seven SPE cartridges tested. From the figure it is clear that C<sub>18</sub> sorbent was the most efficient.  
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### 42 3.1.2. *Effect of conditioning, rinse and elution solvent volume on the SPE extraction*

43 An important parameter in SPE is the breakthrough volume (initial analyte volume) because  
44 enrichment factor depends on the volumes of analyte and volume of eluate. In other words, the  
45 breakthrough volume is the maximum solution volume in which the analytes can be retained  
46 within the acceptable recovery range [17]. As volume of nectar is limited, a sample of 50 μL was  
47 set as the volume to use on the SPE extraction. The amount of sample was reduced to 50 μg  
48 compared with 0.1 g of nectar or 10-100 g used in other studies purposed for honey samples  
49 [18-21]. Remaining this volume constant, the other factors of SPE were tested.  
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57 For column conditioning, different solvent systems were tested: methanol and water and  
58 methanol and acid water (pH 2 with HCl) between 5 to 10 mL of each one. The best results  
59 were obtained with 5 mL of methanol followed by 5 mL of acid water.  
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3 After sample loading, two wash buffers, i.e. water and acid water (pH 2 with HCl) were  
4 investigated. Different volumes of rinse were tested, between 250  $\mu\text{L}$  and 5 mL. It was observed  
5 that 1 mL acid water is sufficient  
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9 Then, 250  $\mu\text{L}$  - 2 mL of methanol and acetonitrile were tested for the elution procedure, it was  
10 observed that 500  $\mu\text{L}$  were adequate to obtain the maximum recoveries.  
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12 Taken together, the conditioning, rinse and elution conditions that provided the highest recovery  
13 values were the following: 1) 5 mL methanol, 2) 5 mL acid water (pH 2), 3) sample loading (50  
14  $\mu\text{g}$  nectar mixed with five parts (1:5, w/v) of acidic water, 4) 1 mL acid water, 5) 500  $\mu\text{L}$   
15 methanol.  
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### 20 21 3.2. Optimization of CE instrumental parameters 22

23 With a view to establishing the best possible compromise between sensitivity, resolution and  
24 analysis time in the separation of all analytes, the following parameters were consecutively  
25 optimized: BGE composition and concentration, injection volume and mode, and other  
26 electrophoretic parameters such as electrophoretic separation voltage, and capillary  
27 temperature and conditioning.  
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33 The buffer pH plays an important role for improving selectivity in CE especially for closely  
34 related compounds, because it affects both the overall charges of the solute and the  
35 electroosmotic flow (EOF). Taking into consideration the acidic nature such of the analytes  
36 under study the effect of the buffer pH was investigated within the range of 8.0/10.0 at a fixed  
37 buffer concentration, adjusted by 0.1 mol L<sup>-1</sup> NaOH and 0.1 mol L<sup>-1</sup> HCl. It was found that  
38 when the pH was lower than 9.0, the resolution was poor. At pH 9.5 baseline separation was  
39 achieved.  
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46 Sodium tetraborate and boric acid were tested as BGEs, but the one producing the best results  
47 considering selectivity, reproducibility, baseline and current performance, was boric acid.  
48 Keeping other parameters constant (pH: 9.5, 25 kV, 25 °C) the buffer concentration was varied  
49 from 10 to 75 mM. Increases in migration times as well as current were observed when the  
50 concentration of buffer increased. Resolution also increased for higher buffer concentrations,  
51 but no appreciable improvements were observed for buffer concentrations above 30 mM. So,  
52 baseline separation was obtained with 30 mmol L<sup>-1</sup> boric acid buffer, pH 9.5.  
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3 The separation voltage affects the electric field strength, which in turn affects the EOF and the  
4 migration velocity of charged particles, which determine the migration time of the analytes. The  
5 effect of the applied voltage was studied over the range 5-35 kV. A potential of 25 kV was found  
6 to provide the best results in terms of run time and resolution between peaks.  
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10 The effect of temperature on electrophoretic separation was examined over the range 15-30 °C.  
11 A temperature of 25 °C was selected as optimal because it provided the best compromise  
12 between migration time (MT) and peak resolution (RS). In fact, raising the capillary temperature  
13 reduced migration times through a decreased electrolyte viscosity, but also led to lower RS  
14 values.  
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17 The injection mode giving the best response concerning reproducibility and linear range was the  
18 hydrodynamic mode. Injection parameters were optimized by varying the lengths of sample (2-8  
19 s) and pressure injection until optimal conditions were reached. The best results were obtained  
20 for the following experimental parameters: hydrodynamic injection mode 30 mbar, 2 s.  
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### 23 *3.3. Repeatability, reproducibility and detection limits*

24 The electropherograms were monitored at 290 nm. A typical electropherogram for the standard  
25 mixture solution under the optimum conditions is shown in Fig. 2. Baseline separation for all  
26 analytes can be achieved within 8 min.  
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29 In order to determine the repeatability of the methodology, replicate injections ( $n = 6$ ) of a  
30 standard mixture solution ( $2.00 \mu\text{g mL}^{-1}$  for each analyte) under the selected optimum conditions  
31 were carried out. The intraday % RSDs of the migration time, corrected area (peak area/tr),  
32 were between 0.8 and 1.6, 1.7 and 3.8, respectively. The interday values for the same  
33 performance criteria were 0.8–1.8 and 1.6–4.9, respectively.  
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36 Calibration curves for the determination of the 12 compounds were constructed under the  
37 optimum conditions. Six concentrations were used for each calibration curve. As shown in Table  
38 1, most of the correlation coefficients of area ratio equations were  $>0.96$ . The corrected peak  
39 area and concentration of each analyte were subjected to regression analysis to obtain the  
40 calibration equations and correlation coefficients, and the results showed that within the  
41 concentration range there was an excellent correlation between the corrected peak area and  
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3 the concentration of each analyte. The limits of detection (LODs) and quantification (LOQs)  
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5 were evaluated on the basis of signal-to-noise ratios (S/N) of 3 and 10, respectively (Table 1).  
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8 In order to determine the accuracy of this method, a 500 µg of nectar (from all the onion lines  
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10 tested) were collected and divided into 10 portions of 50 µg each. The proposed method was  
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12 applied to six portions and the average concentrations determined for each compound were  
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14 taken as a base value. Then, known quantities of the analytes were added to the other aliquots,  
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16 and the phenolic compounds determined following the recommended procedure. The recovery  
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18 studies showed satisfactory robustness leading recoveries higher than 82.00% and lower than  
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20 110.00% for all the analytes under study.  
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### 22 *3.4. Phenolic composition of onion lines*

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24 The optimized CZE method was then applied to determine phenolic compounds in nectar of  
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26 different onion lines. Representative electropherograms are shown in Fig. 3. Markedly  
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28 qualitative and quantitative analytical differences in the phenolic profile of onion lines were  
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30 observed.  
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32 The results of the quantitative determination of phenolic acids and flavonoids are presented in  
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34 Table 2. Of the 12 compounds tested, only 9 were found in the samples. The total amount of  
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36 phenolic compounds ranged between 6.52 and 11.78 mg L<sup>-1</sup>. One of the male sterile lines (MSL  
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38 1) was the nectar with highest phenolic content. Naringenin was found only in male sterile lines  
39  
40 while the open pollinated (OP) line showed the highest amounts of luteolin.  
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42 Phenolics have been reported as insect deterrents or repellents [22-24]. Most of the phenolic  
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44 compounds found in our study are in agreement with those reported in honey; these could be  
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46 explained due to their solubility in aqueous solutions [25]. In a previous work [26], we have  
47  
48 demonstrated that nectar chemical composition has a great influence on bee behavior and seed  
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50 production. Significant differences on chemical composition as well as morphological aspects  
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52 were found between open pollinated and male sterile lines; being male sterile lines the less  
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54 pollinated. From the results shown in Table 2, it can be assumed that compounds such as  
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56 naringenin and luteolin may influence bee foraging behavior, as deterrent and attracting agents,  
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58 respectively.  
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#### 4. Conclusions

At the moment, despite its advantages, to our knowledge there are no reports on the application of SPE-CE-UV for the determination of phenolic compounds in onion nectar. A simplified extraction and rapid CZE method was developed for the isolation and separation of the main phenolic compounds present in onion nectar. Solid phase extraction is used to isolate phenolic compounds from nectar and baseline resolution is achieved in less than 8 min using boric acid buffer system which enabled accurate and reproducible quantification of phenolic compounds in onion nectar. The short analysis time coupled with greatly reduced solvent consumption made it a viable alternative to traditional HPLC. The amount of sample requirement was reduced being up to 2000 times smaller than traditional methods. It has to be pointed out that further studies could greatly benefit from other detection systems such as laser-induced fluorescence and mass spectrometry in order to achieve lower detection limits and identify new phenols in onion nectar.

The markedly qualitative and quantitative analytical differences in the phenolic profile of male sterile and open pollinated lines found in this study contribute to the understanding of the factors that affect onion pollination for hybrid seed production.

#### Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2189), University of Cuyo (6/A445), and National Vegetable Flower and Aromatics Program of INTA. We thank Enza Zaden Seed Company for providing plant material and Héctor Fuligna and Gustavo Lascalea for their valuable support.

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**Table 1. Results of regression analysis on calibration and the detection limits**

Compound	Regression equation $y = a + bx$	Correlation coefficient R	LOD ( $\text{mg L}^{-1}$ )	LOQ ( $\text{mg L}^{-1}$ )
Vinylphenol	$y = 1.8005x - 0.3127$	0.953	0.03	0.11
Rutin	$y = 0.5332x + 0.2383$	0.961	0.6	1.9
Catechin	$y = 0.1732x - 0.0800$	0.926	2.3	7.8
Naringenin	$y = 1.2439x + 0.3912$	0.988	0.1	0.4
Cinnamic acid	$y = 5.2579x + 0.5605$	0.986	0.002	0.006
Chlorogenic acid	$y = 2.3663x - 0.9146$	0.988	0.01	0.04
Quercetin	$y = 2.6086x + 0.9301$	0.985	0.02	0.06
Vanillic acid	$y = 1.3499x - 0.1833$	0.982	0.06	0.2
Luteolin	$y = 3.6397x - 0.3957$	0.983	0.006	0.02
Syringic acid	$y = 2.4795x - 0.6220$	0.987	0.01	0.05
Apigenin	$y = 2.1278x - 0.9225$	0.988	0.03	0.1
Caffeic acid	$y = 3.3751x - 1.1360$	0.967	0.01	0.03

The y and x are the peak current (nA) and concentration of the analytes ( $\text{mg L}^{-1}$ ), respectively. LOD: Limits of detection. LOQ: Limits of quantification.

**Table 2. Phenolic content of different onion lines.**

Compound	Line			
	OP	MSL 1	MSL 2	MSL 3
Vinylphenol	nd	1.17 ± 0.03a	1.1 ± 0.1a	nd
Catechin	nd	nd	nd	2.6 ± 0.1
Naringenin	nd	8. ± 1a	2.6 ± 0.5b	2.5 ± 0.4b
Cinnamic acid	nd	nd	0.57 ± 0.01	nd
Chlorogenic acid	nd	nd	2.1 ± 0.1a	1.84 ± 0.08b
Quercetin	nd	1.04 ± 0.07a	0.19 ± 0.01b	nd
Vanillic acid	0.81 ± 0.03a	0.82 ± 0.03a	nd	nd
Luteolin	7.20 ± 0.2a	nd	nd	0.67 ± 0.03b
Caffeic acid	1.10 ± 0.01a	1.10 ± 0.01a	nd	0.76 ± 0.03b
TPC	9.1 ± 0.2b	12. ± 2a	6.5 ± 0.3c	8.2 ± 0.2bc

OP: open pollinated cultivar. MSL: male sterile line. nd: not detected. TPC: total phenol content. Values represent mean ± SD of 3 determinations. Values in the same column with different letters present significant differences  $P < 0.05$ . Phenolic content is expressed as mg/L of nectar.

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3 **FIGURE CAPTIONS**  
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8 **Figure 1.** Effect of the nature of the sorbents used to pack the minicolumn. Full conditions are  
9 shown in Section 2.

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12 **Figure 2.** Electropherogram of a standard mixture solution ( $2 \text{ mg l}^{-1}$ ). Peaks: 1, vinylphenol; 2,  
13 rutin; 3, catechin 4, naringenin; 5, cinnamic acid; 6, chlorogenic acid; 7, syringic acid; 8, luteolin;  
14 9, apigenin; 10, quercetin; 11, vanillic acid; 12, caffeic acid. Full conditions are shown in Section  
15 2.  
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18 **Figure 3.** CZE profile of phenolic compounds from onion nectar. A: open pollinated cultivar. B:  
19 MSL 1. C: MSL 2. D: MSL 3. Full conditions are shown in Section 2.  
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Fig. 1

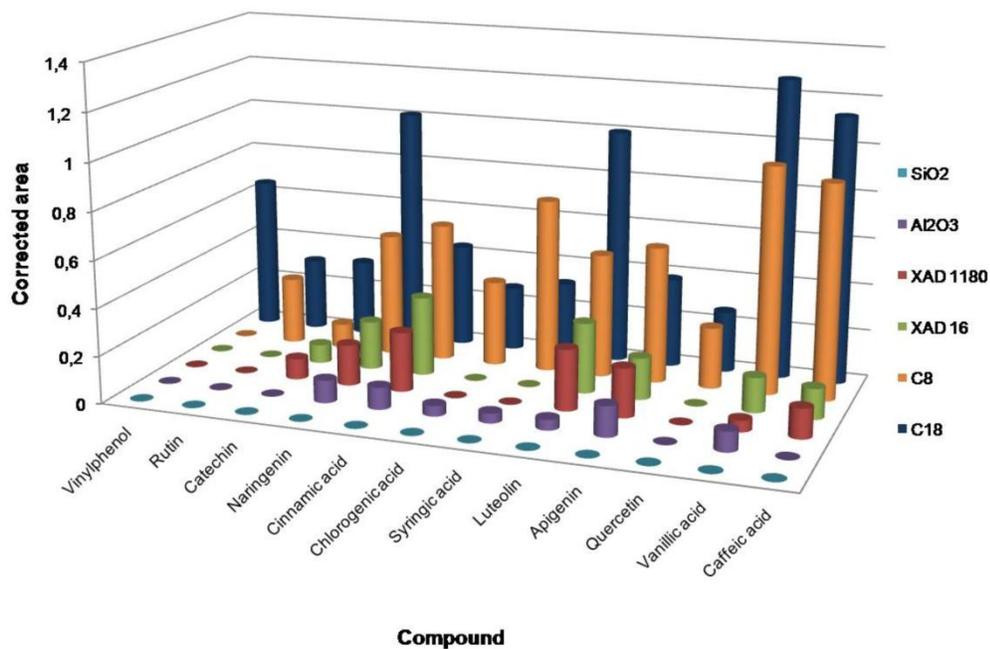


FIG. 2

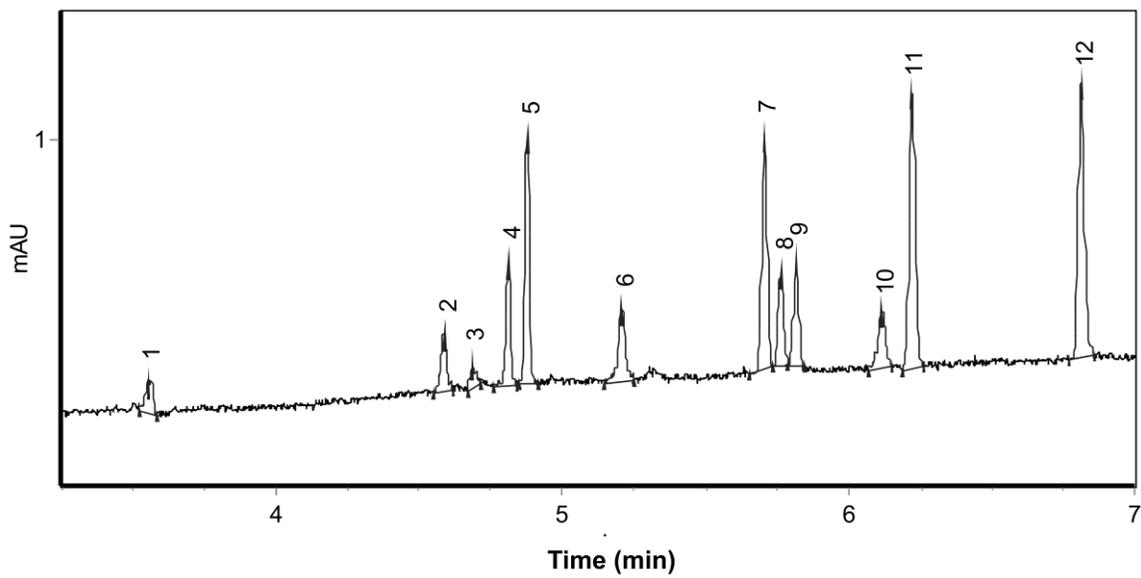
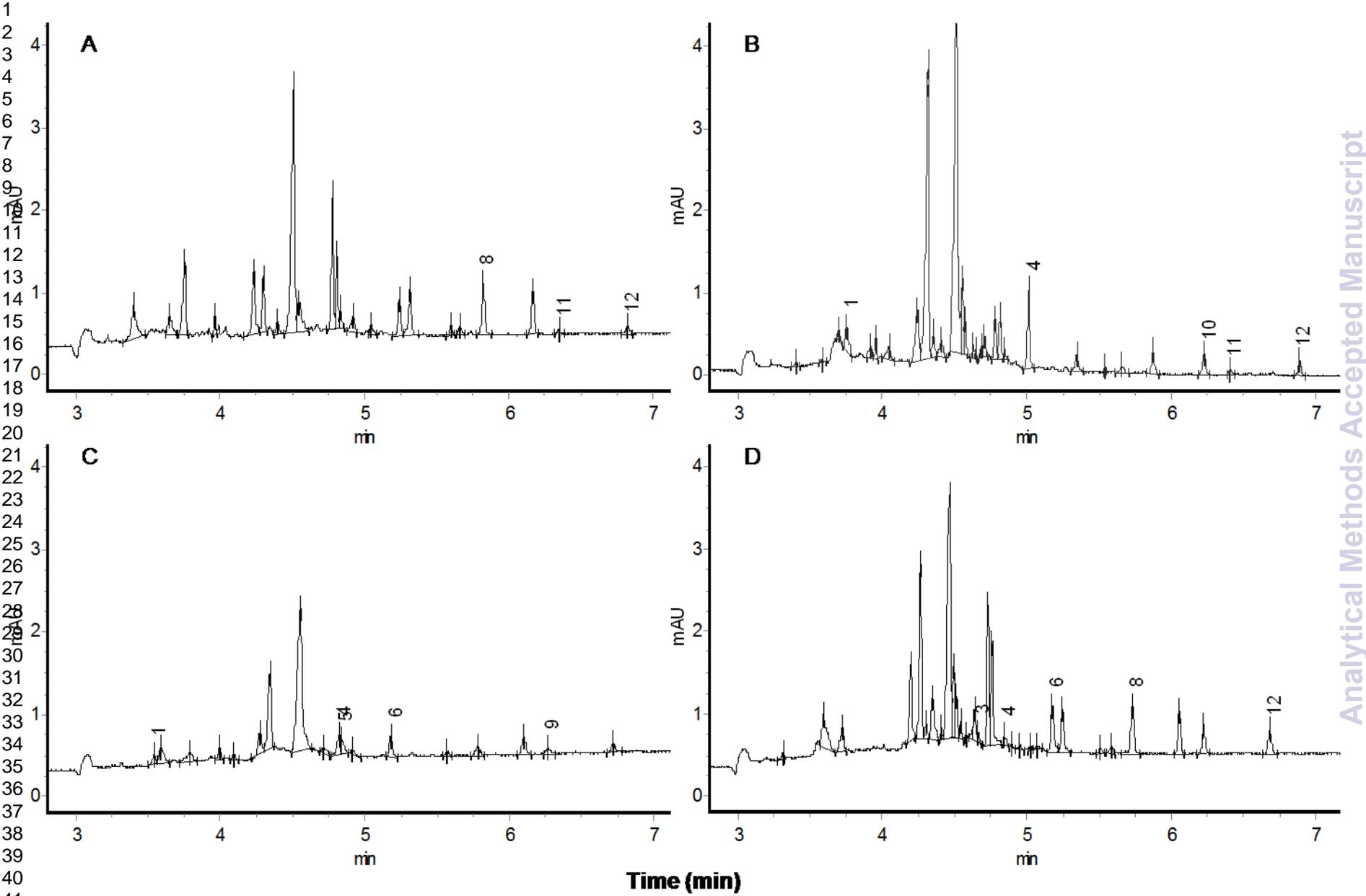


Fig. 3



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