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Research Article

Determination of alkaloids in onion nectar by micellar electrokinetic chromatography

Nectar is the most important floral reward offered by plants to insects. Minor components such as alkaloid compounds in nectar affect bee foraging, with great influence in seed production. CE is an advantageous tool for the analysis of unexplored samples such as onion nectar due to the limited amounts of samples. Considering the importance of these compounds, a simultaneous determination of nicotine, theophylline, theobromine, caffeine, harmaline, piperine in onion nectar by MEKC-UV is herein reported. The extraction of alkaloid compounds in nectar was performed by SPE using a homemade miniaturized column (C_{18}). Effects of several important factors affecting extraction efficiency as well as electrophoretic performance were investigated to acquire optimum conditions. Under the proposed conditions, the analytes can be separated within 15 min in a 50 cm effective length capillary (75 μm id) at a separation voltage of 20 kV in 20 mmol/L sodium tretraborate, 100 mmol/L SDS. The amount of sample requirement was reduced up to 2000 times, when compared to traditional methods, reaching limits of detection as low as 0.0153 ng/L. For the first time, this study demonstrates that there are marked qualitative and quantitative differences in nectar alkaloids between open pollinated and male sterile lines (MSLs) and also within MSLs.

Keywords:
Allium cepa L. / Male sterility / Nectar alkaloids / Seed yield
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1 Introduction

Many plants need animal pollinators to obtain efficient seeds sets. Dicotyledonous plants often attract these pollinators offerings floral nectar that is secreted into the floral tube, located at the base of ovary. Nectar is considered as the main calorific reward by flowers to animal visitors [1]. Nectar is primarily composed of sugars and contains minor compounds such as amino acids, proteins, lipids, phenols, alkaloids, and antioxidants. Some floral nectars can have significant concentrations of ions, such as potassium, especially in onion [2–4]. All these minor components of nectar might directly affect interactions between species and plant health [5].

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 frequently used method for seed production and insects are necessary for pollen transfer [6]. Among insects, honey bees (*Apis mellifera* L.) have been reported to be the most efficient and major onion pollinators, due to their specific instinctive behavioral features that affect both pollen and nectar collection and efficiency in pollen transfer [7]. Consequently, seed yield is closely correlated with the behavior of honey bees in seed onion fields [4].

Two types of onion varieties are usually grown around the world; open pollinated (OP) and first generation (F1) hybrids. In order to produce F1 hybrid seed in onion, it is necessary to cross a male sterile line with a fertile one. In Argentina, onion and garlic are the main fresh vegetables exported [8]. Field observations indicate that F1 hybrid seed yield are much lower than open pollinated varieties seed yields, with a decrease of up to 60% [9, 10]. These differences in yield can be attributed to pollination problems.

Secondary metabolites (SM) are not only found in leaves, but are also found in the floral nectar of plants. SM, including tannins, phenols, alkaloids, and terpenes, have been found in floral nectar [11]. Nectar usually does not repel bees, but a particular nectar may be less attractive than nectar of competing flowers [12]. This so-called toxic nectar is paradoxical given that floral nectar is usually interpreted as attractive, not deterrent, to pollinators. These compounds actually deter bees (*Apis mellifera*) within a wide range of concentrations. The effects of SM on bees are dose- and season dependent. Some alkaloid-containing nectars attract bees in the field even when alternative nectar sources are available. This circumstantial evidence indicates that bees cope with naturally occurring concentrations of SM in nectar. Despite evolutionary and ecological implications, the interaction between bees and SM in nectar has not been widely Studied [13].

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Although alkaloid compounds have been widely studied in different plants, nectars, or fruits, there is a lack of knowledge concerning alkaloids in onion nectar samples. Compounds such as caffeine, nicotine, or methylxanthines have been reported in floral nectar of different species. Nectar has been analyzed in citrus by HPLC-UV, *Datura* species by LC-MS, orange trees by TLC, and tobacco by HPLC [1, 14–16], respectively. To the best of our knowledge, alkaloids in nectar from onion flowers have not been previously analyzed. For the determination of individual compounds, although many traditional sample-preparation methods for alkaloids are still in use, there have been trends in recent years toward: (i) use of smaller initial sample sizes, small volumes, or no organic solvents; (ii) greater specificity or greater selectivity in extraction; (iii) higher recoveries or better reproducibility; and (iv) increased potential for automation. Thus, clean up/preconcentration strategies for the limited amounts of nectar samples by SPE deserve more attention.

On the other hand, CE is becoming increasingly recognized as an important analytical separation technique for the separation and quantification of different compounds due to its speed, efficiency, reproducibility, ultra small sample volume requirements, low cost, and facility for clearing the contaminants. To date, despite the advantages of the application of CE, there are no reports dealing with the determination of alkaloid in onion nectar by this technique.

The classic CZE method is not suited for the separation of alkaloids because of their similarities in charge/mass ratios and limited solubility. MEKC extends the applicability of CE to neutral analytes [17]. In the last decade, indeed, CE methods for the determination of caffeine and other alkaloids have been reported [18–20]. For the neutral characteristics of these molecules precluding any charge-to-mass ratio based separations, usually MEKC methods were proposed [21].

The aim of our work was to develop a sensitive and reliable method for the extraction, separation, and quantification of representative alkaloid compounds in onion nectar (nicotine, theophylline, theobromine, caffeine, harmaline, piperine) by off-line SPE-MEKC-UV. In order to efficiently handle the limited amounts of nectar, homemade SPE cartridges were used. Wide-distributed alkaloids in plants were analyzed within onion flowers (nectar, pollen, and whole flower) for the first time. Differences in the alkaloid contents and distribution of onion lines and/or tissues found in this study may contribute to the understanding of the factors that affect onion pollination for hybrid seed production.

2 Materials and methods

2.1 Plant materials

An open pollinated (OP) onion cultivar, Valcatorce INTA, as well as seven male sterile lines (MSLs), from Enza Zaden, and Seminis were cultivated in a randomized complete block design with three replicates. No pesticides were used along the experiment. The plants flowered from November to December 2014. Onion nectar, pollen, and flowers were sampled at full blossom.

When fruit set was accomplished, umbels were harvested and dried under ambient conditions for 3 weeks. Then, seeds were extracted manually and weighed to estimate seed yield. Relationships between seed yield and frequency of honey bee visits and volatile compounds were estimated.

2.2 Chemicals and reagents

Ultrapure water (resistivity 18.3 MΩ cm) obtained from the Barnstead EASY pure RF water system (Iowa, USA) was used to prepare solutions including the BGE. Nicotine, theophylline, theobromine, caffeine, harmaline, and piperine were purchased from Sigma (St. Louis, MO, USA).

2.3 Solutions and samples

2.3.1 Standard solutions

Standard stock solutions of the analytes were prepared by dissolving an appropriate amount of each pure substance in HPLC-grade methanol to obtain a final concentration of 500 mg/L. The resulting solutions were stored at 4°C in amber glasses. Working standard solutions at a 2 mg/L concentration were prepared on a daily basis by diluting appropriate aliquots of the previous standard stock solutions in buffer. Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3.2 Background electrolyte

Alkaloid compounds were separated in a BGE comprising a 20 mmol/L sodium tretraborate, 100 mmol/L SDS, pH 9.30. All solutions and buffers were degassed by sonication for 5 min.

2.3.3 Sample treatment and SPE procedure

In order to obtain the nectar in the most natural way, and preserve it under similar conditions as it is in the plant, the most effective way of extraction was to separate freshly opened flowers from umbels, removing anthers, filaments and peduncle, and immediately centrifuging (13 000 rpm, 30 min, 4°C) in a 1.50 mL microtube. It was possible to extract around 10 μL of nectar from each umbel. Pollen, from OP onion, was separated from stamens, dried at room temperature, and transferred into an Eppendorf tube. Flowers were freeze-dried during blossom. Then, all materials stored at −80°C until analyzed.

Nectar samples (50 mg) were thoroughly mixed with three parts (1:3, w/v) of water (pH 2.00), with HCl until complete homogenization. Pollen and flower samples (50 mg)

were suspended in 500 μL of water (pH 2.00). After a 30-min sonication at 30°C and 15 min of centrifugation samples were filtered through a membrane.

The extraction of alkaloid compounds in nectar, pollen, and flower samples was performed by SPE using a homemade column packed with a suitable filtering material. C_{18} cartridges (50 mg) were made in 1 mL syringes using 25 mg of glass wool as frits. These cartridges were placed in a vacuum elution apparatus (Varian Vac Elut 20 manifold and a Vacuubrand vacuum pump ME 2C) and preconditioned by passing 5 mL of methanol and 5 mL of water pH 2.00, with HCl. Samples (50 mg) were carefully loaded onto the preconditioned column, driving the sample through the solid phase using vacuum. Then, the column was washed with 1 mL of water pH 2.00, with HCl. The alkaloid compounds present in nectar remained in the column while sugars and other highly polar compounds were eluted with the aqueous solvent. The whole alkaloid fraction was eluted with methanol (500 μL). The eluent was directly injected and analyzed by MEKC.

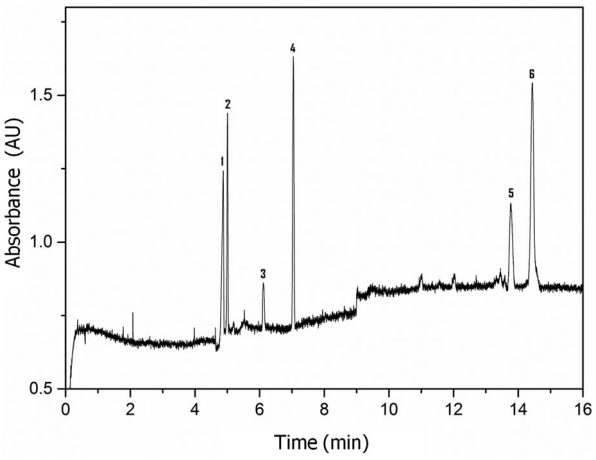

Figure 1. Electropherogram of a standard mixture solution (0.5 mg/L). Peaks: 1, caffeine; 2, theobromine; 3, nicotine 4, theophylline; 5, piperine; 6, harmaline. Full conditions are shown in Section 2.

2.4 Micellar electrokinetic chromatography

MEKC was carried out using a Capel™105M apparatus equipped with a 57 cm full length, 50 cm effective length, 75 μm id, and 375 μm od fused silica capillary. The capillary tube was conditioned prior to its daily use by flushing with water (5 min), 0.10 mol/L NaOH for 5 min, followed by water for another 5 min, and finally with the buffer for 3 min. The running buffer was 20 mmol/L sodium tretraborate, 100 mmol/L SDS, pH 9.30. The separation voltage was 20 kV and the capillary temperature was 25°C. Samples were injected by hydrodynamic injection at 30 mbar for 3 s. Electropherograms were recorded at 220 nm. Between runs, the capillary was flushed with water (3 min), 0.10 mol/L NaOH (2 min), water (2 min), and fresh buffer (2 min). The capillary tube was rinsed with 0.10 mol/L 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 the 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 MEKC parameters

As already mentioned, the classic CZE method is not suited for the separation of alkaloids due to their poor solubility and that most of them are not ionizable under working pH conditions (i.e. $pKa_{caffeine} = 14$). Consequently, caffeine would co-elute with any neutral compound present in the sample. The micellar "pseudostationary" phase in MEKC interacts with the analytes according to partitioning mechanisms, just like in a chromatographic method. In order to establish the best possible compromise between sensitivity, resolution, and analysis time in the separation of all analytes, the following parameters were consecutively optimized: BGE composition and concentration, injection volume and mode, and other electrophoretic parameters such as electrophoretic separation voltage, and capillary temperature and conditioning. The first parameter studied during method development was the BGE composition and its concentration. Boric acid, phosphoric acid, potassium phosphate monobasic, and sodium tetraborate were tested. Buffer concentracions of 20 mmol/L, 50 mmol/L, and 75 mmol/L were mixed with different SDS concentrations (10–100 mmol/L, with the addition of methanol (0–10%) (v/v). The effect of the buffer pH was investigated within the range of 3.00–12.00, adjusted by 0.50 mol/L HCl or 0.50 mol/L NaOH, respectively. It was found that when the pH was lower than 8.0, the resolution was poor. Increases in migration times as well current were observed when the concentration of buffer increased. Resolution also increased for higher buffer concentrations, but no appreciable improvements were observed for buffer concentrations above 20 mM. Increases of SDS concentrations greatly improved separation as well as peak shape; a higher number of micelles result in a larger retention of analytes. Baseline separations were obtained for SDS concentrations higher than 90 mM. On the other hand, the addition of organic modifier did not improve separation efficiency. Considering selectivity, reproducibility, baseline and current performance, the best results were obtained when 20 mmol/L sodium tetraborate and 100 mmol/L SDS was used at pH 9.30.

Table 1. Results of regression analysis on calibration, detection, and quantification limits

Compound	Regression equation y = a + bx	Correlation coefficient R	LOD (ng/L[1])	LOQ (ng/L[1])	tm
Caffeine	y = 29.363x + 1.6745	0.9980	0.1190	0.3967	4.873
Teobromine	y = 22.059x + 2.2515	0.9950	0.3413	0.4233	5.007
Nicotine	y = 7.4486x + 0.9422	0.9919	1.1760	4.5522	6.115
Teophylline	y = 40.538x + 3.2291	0.9985	0.0153	0.0513	7.043
Piperine	y = 20.544x + 5.3544	0.9885	0.1752	0.6501	13.772
Harmaline	y = 75.069x − 3.2306	0.9969	0.0599	0.0163	14.432

The y and x are the peak current (nA) and concentration of the analytes (ng/L), respectively. tm, migration time in minutes.

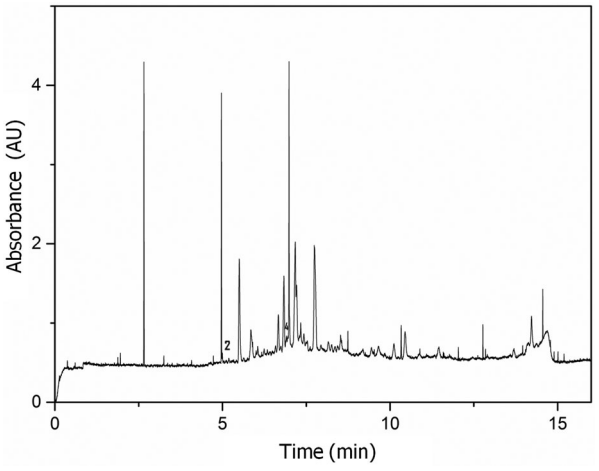

Figure 2. MEKC profile of alkaloid compounds from an onion nectar sample (MSL 3). Peaks: 2, theobromine; 4, theophylline. Full conditions are shown in Section 2.

The effect of the applied voltage was studied over the range 15–25 kV. Based on experiments, 20 kV was chosen as the optimum voltage to accomplish a good compromise, in terms of run time and resolution. The effect of temperature on electrophoretic separation was examined over the range 15–30°C. In fact, raising the capillary temperature reduced migration times through a decreased electrolyte viscosity, but also led to lower RS values. No appreciable improvements were observed for temperatures above 20°C. A temperature of 20°C was selected as optimal because it provided the best compromise between migration time (MT) and peak resolution (RS).

The injection mode giving the best response concerning reproducibility and linear range was the hydrodynamic mode. Injection parameters were optimized by varying the time (2–6 s), and pressure (10-30 mbar), until optimal conditions were accomplished. Since the injection time determining the amount of sampling affects both peak area and peak shape, the best results were obtained for the following experimental parameters: hydrodynamic injection mode 30 mbar, 3 s.

Taking into account the optical properties of the selected analytes, wavelength detection was varied within the range 200–280 nm. A 220 nm wavelength detection was chosen considering the differences in sensitivity, linear range, and concentration range of the analytes in the different samples.

A typical electropherogram for the standard mixture solution under the optimum conditions is shown in Fig. 1. As it can be observed, baseline separation for all analytes was achieved in less than 15 min.

3.2 Sample clean-up

Taking into account that nectar is a salty-aqueous matrix composed mainly by sugars, up to 80% an extraction step was necessary to avoid matrix effects. Several variables were tested to determine the most suitable conditions for the extraction of alkaloid compounds in onion flowers. The yield and repeatability of the extraction were affected by factors such as the type of sample volume, solvent, or dilution. In this step of method development, the real samples of nectar were investigated, peak areas being the analytical response evaluated. The variation coefficients (CV) for optimization of extraction conditions were calculated as relative standard deviations of the corrected area (peak area/tr) for the triplicate analyses of real samples.

Different procedures for alkaloid extraction were tested in nectar, pollen, and flower samples. Taking in account previous methods of alkaloid extraction in these kind of samples [1,14–16], samples were diluted in methanol, water, BGE, and ethanol 1:5 (w/v). Equilibration times (10–60 min) with or without sonication were evaluated. Results were not satisfactory for any of the evaluated procedures. Thus, a sample preconcentration was needed.

A previous SPE procedure developed and optimized to isolate and preconcentrate the phenolic fraction in the nectar samples using a homemade column [10] was considered. The following parameters were evaluated: including the volume and composition of the conditioning and eluting solvents, and chemical nature and amount of the sorbents. Three kinds of bonded silica sorbents: C8 (particle size: 56 μm), C18 (55 μm), and Strata-X (28–34 μm). The best results were obtained for the following conditions. Nectar samples (50 mg) were thoroughly mixed with four parts (1:4, w/v) of water (pH 2.00), with HCl until complete homogenization and carefully loaded onto the preconditioned column, leaving the sample on the solid phase under vacuum. Then, the column was washed with 1 mL of water (pH 2.00), with HCl. The alkaloid compounds present in nectar remained in the column while sugars and other highly polar compounds were eluted with the aqueous solvent. The whole alkaloid

Table 2. Alkaloid content in different onion lines[a)]

Compound	Line							
	OP	ML1	ML2	ML3	ML4	ML5	ML6	ML7
Caffeine	nd	Nd	nd	nd	27.59 ± 1.68	nd	nd	nd
Teobromine	197.44 ± 2.89 b	170.10 ± 4.97 cd	173.41 ± 13.26 c	69.27 ± 5.80 f	34.28 ± 5.09 g	157.13 ± 4.64 de	144.50 ± 4.78 e	219.01 ± 13.31 a
Nicotine	nd	Nd	nd	nd	nd	nd	nd	nd
Teophylline	551.83 ± 16.62 a	333.96 ± 28.54 d	nd	479.30 ± 33.41 b	406.46 ± 13.47 c	184.77 ± 10.21 f	137.83 ± 11.32 g	237.49 ± 28.54 e
Piperine	nd	Nd	nd	nd	nd	nd	nd	nd
Harmaline	nd	Nd	nd	nd	nd	nd	nd	nd

[a)] OP, open pollinated cultivar

MSL, male sterile line; nd, not detected. Values represent mean ± SD of three determination steps. Values in the same file with different letters present significant differences $p < 0.05$. The alkaloid content is expressed as nanogram per liter of nectar.

fraction was eluted with methanol (500 μL). Then, the eluate was dried under a stream of N_2 and the residue dissolved, for MEKC, in 300 μL BGE. Pollen and flowers (50 mg) were mixed with four parts (1:10, w/v) of water (adjusted to pH 2.00), sonicated for 30 min, and centrifuged for 15 min at 13 000 rpm. The supernatant was extracted likewise nectar.

3.3 Repeatability, reproducibility, and detection limits

In order to determine the repeatability of the methodology, replicate injections ($n = 6$) of a standard mixture solution (2.00 μg/mL for each analyte) under the selected optimum conditions were carried out. The intraday percent RSDs of the migration time, corrected area (peak area/tr), were between 1.42 and 4.02, 0.75 and 6.25, respectively. The interday values for the same performance criteria were 0.99–3.75 and 2.14–6.20, respectively.

Calibration curves for the determination of the six compounds were constructed under the optimum conditions. Six points of the calibration curve were determined (three technical replicates at each concentration level). The calibration equations were calculated by the least-squares linear regression method. Thus, linearity was evaluated from values closer to the LODs values up to approximately 10 mg/L.

The corrected peak area and the concentration of each analyte were subjected to regression analysis to obtain the calibration equations and correlation coefficients. The LODs and LOQs were evaluated on the basis of S/N of 3 and 10, respectively (Table 1). The calibration graphs were linear at levels near the detection limits up to at least 50 mg/L.

3.4 Matrix effects

In order to determine the matrix effect over each analyte response, calibration curves from a spiked matrix and spiked pure solvent samples were created. Thus, calibration curves from spiked matrix and spiked pure solvent samples were created for each analyte. The percentage of the quotient of the slopes (b) in the spiked and solvent samples was used as an indicator of matrix effect, which were calculated as shown in Eq. (1).

$$Matrix\ Effect\,\% = -\left[\left(\frac{b_{spiked}}{b_{solvent}}\right) \times 100\right] \quad (1)$$

Matrix effects for the alkaloids under study were within the range: 1.80–5.60%. The latter demonstrated the efficiency of the proposed SPE/MEKC approach; all slope ratios were close to one (1.00 ± 0.03). Thus external calibration was chosen.

3.5 Method validation

In order to determine the accuracy of this method, 500 mg of nectar (from all the onion lines tested) were collected and divided into ten portions of 50 mg each. The proposed method was applied to six portions and the average concentrations determined for each compound were taken as a base value. Then, known quantities of the analytes were added to the other aliquots, and the alkaloid compounds were determined following the recommended procedure. The recovery studies showed satisfactory robustness leading to recoveries higher than 82.00% and lower than 110.00% for all the analytes under study.

3.6 Alkaloids in onion lines

In a first approach the optimized MEKC method was then applied to determine alkaloid compounds in nectars of different onion lines. A representative electropherogram is shown in Fig. 2. Markedly qualitative and quantitative analytical differences in the profile of onion lines were observed. The results of the quantitative determination of alkaloid compounds in nectar onion lines are presented in Table 2. Out of the six compounds tested, only three were found in the samples. Theophylline was the main alkaloid, accompanied by theobromine, while caffeine occurred in traces only in one MLS nectar sample.

Likewise,differences were observed among nectar, pollen, and flower samples of the OP line (Fig. 3). Signifi-

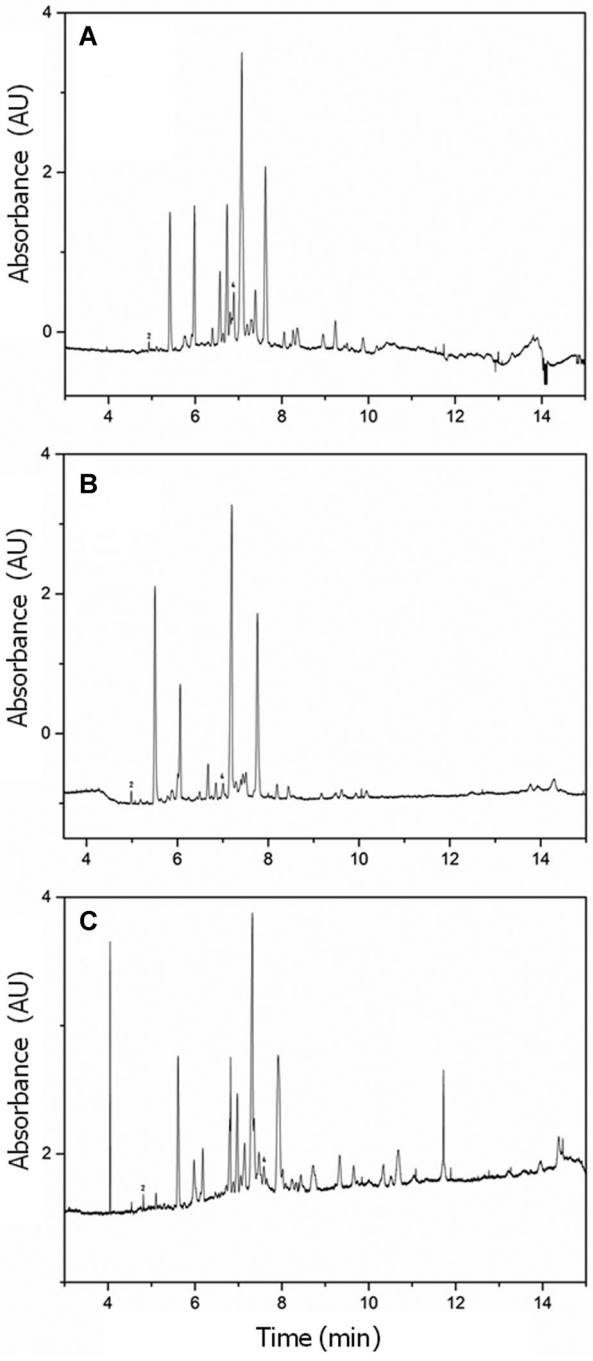

Figure 3. Profile of alkaloid compounds from (A) flower; (B) pollen; (C) onion nectar. Peaks: 1, caffeine; 2, theobromine; 4, theophylline.

cant difference was found between ($p < 0.05$) among nectar, flower, and pollen theobromine concentration (Table 3).

Until now the presence of theophylline and other methylxanthines in onion flowers or nectar has not been reported. However, our results are in agreement with those reported by Kretschmar and Baumann 1999, who found that these alkaloid compounds are found in a higher concentration in pollen than nectar in citrus flowers. A future study of the purine alkaloids within the MLS that do not have viable pollen grains would be needed.

Table 3. Alkaloid content in nectar, pollen, and flower of the open pollinated onion line

Compound	OP Line		
	Nectar	Pollen	Flower
Caffeine	nd	nd	nd
Teobromine	197.44 ± 2.89 c	1208.06 ± 21.63 a	695.89 ± 30.48 [b]
Nicotine	nd	nd	nd
Teophylline	551.83 ± 16.62 c	1144.55 ± 4.13 b	1639.05 ± 89.56 [a]
Piperine	nd	nd	nd
Harmaline	nd	nd	nd

[a)] OP, open pollinated cultivar.
nd, not detected. Values represent mean ± SD of three determination steps. Values in the same file with different letters present significant differences $p < 0.05$. The alkaloid content is expressed as nanogram per liter, nanogram per gram for nectar and pollen or flower, respectively.

In previous studies we have demonstrated that nectar chemical composition has a great influence on bee behavior and seed production [4, 10, 22]. Alkaloids have been reported as insect deterrents or repellents [5, 23, 24]. Nevertheless, we have not found a direct relationship between these compounds and seed production (data not shown). Interestingly, the higher concentrations of methylxanthines were found in the OP samples, the line with highest seed yield. The latter suggest that from the pollinator's perspective, the attractiveness of plants is determined primarily by the perceived amount of beneficial compounds such as carbohydrates and amino acids contained in floral nectar. Ecological context should thus be considered when assessing ecological costs of plant defense in terms of pollination services [25]. Köhler et al. 2012 [24], reported that a dose-dependent deterrent effect of nicotine was stronger in lower sugar concentrations, but even the highest nicotine concentrations did not completely repel honeybees, i.e. bees did not stop feeding on these diets. Likewise, bee behavior could be affected by methyxantines.

4 Concluding remarks

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. Nevertheless, widespread use of the honeybee as pollinator, not always bring about the expected results because the onion nectar is not particularly attractive for bees. Our group has demonstrated the presence of deterrent compounds in onion nectar, as well as important differences in seed production as a result of selective foraging behavior due to the presence of such compounds. Although volatile and phenolic compounds have already been studied, there is lack of information about alkaloids in onion nectar.

This is the first report describing the application of CE for the determination of alkaloid compounds in nectar. A simplified extraction and rapid MEKC methodology was developed for the isolation and separation of the alkaloid compounds present 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 up to 2000 times compared to traditional methods. Our work stands for advance in the analytical knowledge of alkaloids in an unexplored sample such as nectar and thus contributes with novel, simple, low cost, and green analytical tools for the understanding of pollination in a crop of great nutritional and economical potential. It has to be pointed out that further studies could greatly benefit from other detection systems in order to achieve lower detection limits and identify new alkaloids in onion nectar.

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The authors have declared no conflict of interest.

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