Marina Arias[1]*
Carolina Simó[2]*
Luis T. Ortiz[3]
Marcelino de los Mozos-Pascual[1]
Coral Barbas[4]
Alejandro Cifuentes[2]

[1]CIA de Albaladejito, Cuenca, Spain
[2]Inst. Industrial Fermentations (CSIC), Madrid, Spain
[3]Facultad de Veterinaria, Univ. Complutense, Madrid, Spain
[4]Facultad de CC. Experimentales y Técnicas, Universidad San Pablo-CEU, Madrid, Spain

Detection and quantitation of a bioactive compound in *Vicia narbonensis* L. seeds by capillary electrophoresis-mass spectrometry: A comparative study with UV detection

Capillary zone electrophoresis with mass spectrometry (CE-MS) and UV detection (CE-UV) was applied to the quantitative determination of γ-glutamyl-*S*-ethenyl-cysteine (GEC), a bioactive and unstable compound present in *Vicia narbonensis* L. seeds. This compound is responsible for, among other negative effects, palatability reduction and grain toxicity. In order to carry out the quantitative analysis of GEC, different conditions (such as composition, concentration and pH of the background electrolyte, and type and time of extraction) were studied. Also, adequate conditions for electrospray-mass spectrometry of this bioactive compound were investigated. The best extraction conditions of GEC from *V. narbonensis* L. seeds flour were obtained using ethanol-water (70:30 v/v) for 45 min. The use of a 20 m ammonium hydrogen carbonate at pH 7 provided adequate analytical conditions compatible with the unstable nature of GEC as well as with the requirements of CE-UV and CE-MS analysis. A comparative study was carried out between the different figures of merit of CE-UV and CE-MS for quantitative purposes. Both techniques provided similar limit of detection and can be applied with confidence within the same linear dynamic range. However, reproducibility and speed of analysis were better using CE-UV. The developed methods were readily applied to quantify GEC in seeds of 21 genotypes of *V. narbonensis* L.. A good agreement between CE-MS and CE-UV results was observed corroborating the usefulness of both approaches for quantitative purposes.

Keywords: Antinutritional factors / Capillary electrophoresis / γ-Glutamyl-*S*-ethenyl-cysteine / Mass spectrometry / Peptides / *Vicia narbonensis* DOI 10.1002/elps.200410224

1 Introduction

Currently, obtaining new sources of suitable protein for animal feeding is taking an important place in animal feeding research. Legumes have traditionally been used as a vegetable protein source in human and livestock diets, but some of them have almost been consigned to oblivion due to their high content in antinutritional factors. Among these legumes, we can find the species *Vicia narbonensis* L., commonly called Narbon bean. This legume plant also has very good agronomic and nutritive qualities (the reason that turns it into our aim of study), and appears as "*a leguminous species with the potential to become an important grain and straw crop for animal feed in dry temperate areas*" [1].

V. narbonensis, a relative of *Vicia faba* L. [2], is originally from Mediterranean regions. Some interesting features of this species are its erect growth habit, taproot system, low incidence of disease and pest attack, good adaptation to alkaline soils and low rainfall areas [3], and the ease of sowing and harvesting. Narbon bean plants can be grazed by sheep or cattle and are also suitable for production of high-quality hay. The grain can also be used as a supplement for feeding sheep. However, it is not recommended for feeding nonruminant livestock due to their inability to detoxify it [2]. Because of its resistance to cereal diseases and multiple end uses (grain, grazing, conserved fodder and green manure), it could play a valuable role in rotation [4]. It has been proven that this grain legume adapts very well to many locations with different edaphic and climatic conditions and produces a good seed yield (*e.g.,* 1.0–2.3 t/ha in Australia) [5].

Correspondence: Dr. Alejandro Cifuentes, Inst. Industrial Fermentations (CSIC), Juan de la Cierva 3, E-28006 Madrid, Spain
E-mail: acifuentes@ifi.csic.es
Fax: +34-91-5644853

Abbreviation: GEC, γ-glutamyl-*S*-ethenyl-cysteine

* These two authors contributed equally to this work.

Among the different components of *V. narbonensis,* special attention should be paid to the sulphur-containing dipeptide γ-glutamyl-*S*-ethenyl-cysteine (GEC), a bioactive compound in seeds in a range of 0.41 to 3.77% [2]. GEC dipeptide is responsible for palatability reduction in grain and some of the toxic properties for nonruminants. It can also reduce intake and growth rate of broiler chickens and pigs [2] and has a negative effect in the production of sheep wool when they are fed *V. narbonensis* [6]. Cattle appear to tolerate it better, but the milk should be treated, due to its bitter taste caused by the Narbon beans [7]. The GEC dipeptide has an 11.6% sulphur content, which means *ca.* 30% of the total sulphur in the seed. This sulphur content, due to dipeptide, varies with genotype and environment, with up to 0.3% in dry weight (DW) of the seeds in some cases [8].

In spite of the importance of this GEC dipeptide, very few procedures have been developed so far to analyze this compound. Thus, analysis of GEC with HPLC technique seems to be not possible due to the unstable nature of this compound in strongly alkaline conditions used for *O*-phthaldehyde (OPA) derivatization and in the presence of deproteination reagents [6]. CE techniques seem to be more appropriate for analyzing this compound since it is possible to maintain pH conditions close to neutral, minimizing any breakdown of GEC [6]. However, to carry out this type of analysis it is necessary to account with GEC standard, and, to our knowledge, this compound is not commercially available. So far, the usefulness of CE to analyze this dipeptide was demonstrated by Enneking *et al.* [8], although in their elegant work no electropherograms were given. Moreover, the MEKC method was used to analyze the crystalline GEC dipeptide previously purified and derivatized with 9-fluorenylmethoxycarbonyl (FMOC) [8].

CE coupled with MS represents an attractive analytical method due to the high specificity and structural information that can be obtained [9]. Thus, CE-MS coupling using ESI has given rise to a powerful analytical technique able to combine the advantages of these two procedures [10–12]. In spite of these advantages, CE-MS is rarely used to analyze food and feed compounds [13], however, CE-MS would be highly suitable for GEC analysis, since it presents a structure comparable to small peptides and amino acids [14, 15]. It has to be emphasized that the CE mode used in reference [8] to analyze the crystalline GEC dipeptide was MEKC, using a borate-SDS-based buffer, which is incompatible with CE-MS analysis.

The goals of this work were, therefore, to (i) develop a new and fast CE method compatible with CE-MS, and (ii) to directly analyze the bioactive compound GEC from *V. narbonensis* L. seeds. Moreover, a comparison is established between the quantitative capabilities of CE-MS and CE-UV when applied to determine this solute.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogen carbonate from Fluka (Buchs, Switzerland), ammonium hydroxide from Merck (Darmstadt, Germany) and formic acid from Riedel-de Häen (Seelze, Germany) were used for the CE running buffers at different concentrations and pHs. Buffers were prepared by weighing the ammonium hydrogen carbonate at the concentrations indicated in each case and adding 1 M ammonium hydroxide or formic acid to adjust the pH. The buffers were stored at 4°C and warmed at room temperature before being used. Methanol HPLC-grade and ethanol, both from Scharlau (Barcelona, Spain), were used for sheath liquid preparation and seed flour extraction procedure, respectively. SDS from Fluka and sodium hydroxide from Panreac (Barcelona, Spain) were used for the capillary cleaning procedure before each analysis. Purified GEC dipeptide was a gift from Dr. Max E. Tate from the University of Adelaide (Australia), and it was directly prepared by dissolving in an ethanol-water (70:30 v/v) dilution to obtain a final concentration indicated in each case. Water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Samples

Twenty-one *V. narbonensis* genotypes were analyzed to determine their GEC content. Twenty of them were collected in different Spanish and Italian regions (as shown in Table 1) and one commercial cultivar called Altair was a gift from AGROSA, Semillas Selectas (Jadraque, Guadalajara, Spain). All of them were multiplied during the 2001–2002 season in trial fields of the Centro de Investigación Agraria de Albaladejito, placed in Cuenca (Spain) and stored at the Regional Vegetable Germ Plasm Bank of Albaladejito until 2004. Seeds were ground with an Ultra Centrifugal mill ZM 1000 from Retsch (Newtown, PA, USA) (1 mm sieve size) and stored at 4°C in plastic bags.

2.3 Extraction of GEC

Several extraction conditions were developed in order to maximize GEC recovery from the seed flour samples. For this purpose, different ethanol percentages (100, 90, 80, 70, 60, 50, 40, and 0%) and extraction times (45 min, 2 h,

Table 1. Passport data and GEC contents of Narbon bean accessions (passport data provided by Centro de Recursos Fitogenéticos CRF-INIA, Madrid, Spain)

Sample No.	CRF No.	Country of origin	Collecting year	Collecting area (country)	Altitude (m)	GEC_{UV}[a] (%)	RSD[b] (%)
1	BGE019584	Spain	1987	Andalucía (Spain)	180	2.28	0.9
2	BGE005512	Spain	1983	Andalucía (Spain)	826	2.21	1.2
3	BGE029694	Spain	1998	Madrid (Spain)	1203	2.43	1.2
4	BGE025291	Spain	1996	Andalucía (Spain)	794	2.25	1.9
5	BGE013235	Spain	1985	Andalucía (Spain)	866	2.45	1.4
6	BGE001571	Spain	1978	Andalucía (Spain)	50	2.27	1.8
7	BGE013237	Spain	1988	Castilla-La Mancha (Spain)	527	2.12	0.3
8	BGE011729	Spain	1980	Andalucía (Spain)	402	2.41	2.0
9	BGE029056	Spain	1997	Andalucía (Spain)	891	2.62	0.3
10	BGE023509	Spain	1995	Madrid (Spain)	587	3.06	1.6
11	BGE009982	Spain	1980	Castilla-La Mancha (Spain)	529	1.57	1.4
12	BGE009987	France Germany	1985	Andalucía (Spain)	568	2.07	1.9
13	BGE013236	Spain	1987	Andalucía (Spain)	694	2.35	1.4
14	BGE013234	Spain	1985	Castilla-La Mancha (Spain)	669	2.13	1.8
15	BGE001894	Spain	1978	Castilla-La Mancha (Spain)	725	1.55	1.6
16	BGE018826	France Germany	1985	Castilla-La Mancha (Spain)	529	2.28	0.4
17	BGE022216	Spain	1992	Baleares (Spain)	142	2.39	1.4
18	BGE022759	Italy	1986	Sicilia (Italy)	240	2.65	0.5
19	BGE019585	Spain	1987	Andalucía (Spain)	160	2.43	0.6
20	BGE031093	Spain	1999	Andalucía (Spain)	794	1.89	0.4
21	ALTAIR[c]	–	–	–	–	2.01	1.5

a) GEC (%) (dry weight) determined by CE-UV in this work (see text).

b) RSD (%), (n = 3) calculated as follows: $\mathrm{RSD}(\%) = \frac{s}{\bar{y}} \cdot 100$ where $s = \sqrt{\frac{\sum (y_i - \bar{y})^2}{n-1}}$.

c) Commercial seed (see section 2.2, Samples).

and 24 h) were tested. Optimum extraction conditions consisted of the addition of 1 mL of ethanol-water (70:30 v/v) to 0.1 g of seed flour, followed by stirring for 45 min at 4°C in darkness. Extracts were then centrifuged at 8315 × *g* for 10 min at 5°C, to remove all undesirable solid particles. Supernatant was directly injected in CE. Several ultrasound experiments were carried out in order to test the possibility of improving the recovery of the GEC dipeptide from seed flour. Namely, the extraction methods tested consisted of the procedure described above plus ultrasounds for 5, 10, and 15 min (for a total extraction time of 45 min).

2.4 CE

Optimum CE-UV conditions were studied using a P/ACE 2050 (Beckman Instruments, Fullerton, CA, USA) apparatus, equipped with an UV-Vis detector working at 200, 214, and 254 nm. Bare fused-silica capillaries with 50 μm ID were purchased from Composite Metals Service (Worcester, England). The detection lengths used for CE-UV analysis were 30 cm (total length, 37 cm) or 20 cm (total length, 87 cm). Injections were made at the anodic end using N_2 pressure of 0.5 psi (1 psi = 6894.76 Pa) during a given time. Different separation buffers were tested (see below). The buffer selected was 20 mM ammonium hydrogen carbonate at pH 7. Buffer was prepared by dissolving 0.158 g of ammonium hydrogen carbonate in 95 mL water, adjusting till pH 7 with formic acid and adding water till a final volume of 100 mL. Separation was performed at 15 kV and 25°C. In order to eliminate adsorbed compounds from flour extracts onto the capillary wall, 0.1 M NaOH with 50 mM SDS solution was used to clean it prior to each run (1.5 min using N_2 pressure of 20 psi). After the cleaning procedure, conditioning was completed by flushing the capillary (at 20 psi) with water and running buffer for 1.5 and 2 min, respectively. The P/

ACE 2050 instrument was controlled by a PC running the System Gold software (Beckman). For CE-MS analysis, a P/ACE 5010 (Beckman) CE apparatus equipped with a UV-Vis detector working at 200 nm was coupled to the MS detector using an orthogonal electrospray interface (see below). Bare fused-silica capillaries with 50 μm ID, 20 cm UV detection length, and 87 cm MS detection length, were used. Injections were made at the anodic end using N_2 pressure at 0.5 psi for 12 s, with the nebulizer gas turned off. The P/ACE 5010 instrument was controlled by a PC running the System Gold software (Beckman). All new capillaries for CE-UV or CE-MS were conditioned by flushing with 0.1 M NaOH for 20 min, followed by water for 30 min.

2.5 MS

CE-MS experiments were performed on an Esquire 2000™ ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Electrical contact at the electrospray needle tip was established *via* a flow of conductive sheath liquid, which consisted of methanol-water (50:50 v/v) containing 0.1% v/v acetic acid, and delivered at a flow rate of 3 μL/min by a 74900–00–05 Cole Palmer syringe pump (Vernon Hills, IL, USA). The mass spectrometer was operated in the positive ion mode. The spectrometer was scanned at 200–400 *m/z* range at 13 000 u/s during separation. MS operating conditions were optimized by adjusting the needle-counter electrode distance, capillary position [16], nebulizer pressure value, and liquid sheath flow rate and composition, while a GEC sample was injected and separated in the CE-ESI-MS system. The optimum nebulizer/drying gas conditions were: 11 psi nitrogen, and 8 L/min nitrogen at 120°C. The instrument was controlled by a PC running the Esquire NT software (Bruker).

3 Results and discussion

As indicated above, a new separation buffer compatible with CE-UV (and mainly with CE-MS) had to be found for GEC analysis. Also, we have observed in various literature that authors have tried different conditions for extracting GEC from seeds [6, 8], varying in composition of extracting solutions (*e.g.,* the composition of dissolutions varied from 30 to 60% ethanol) and time of extraction (from 1.5 to 24 h). Therefore, we decided to test different extraction conditions as well.

3.1 Developing a new method to extract GEC from seeds

Several extraction conditions were tested in order to maximize GEC recovery from seed flour samples. Namely, different ethanol percentages (100, 90, 80, 70, 60, 50, 40, and 0%) and extraction times (45 min, 2 h, and 24 h) were tested. These extracts were used together with different volatile CE buffers for GEC analysis. It was observed that the best extraction conditions consisted of the addition of 1 mL of ethanol-water (70:30 v/v) to 0.1 g of seed flour, followed by stirring for 45 min (see below). In order to be sure that after 45 min we have arrived to the complete extraction of GEC, an additional extracting procedure was tested using ultrasounds for 5, 10, and 15 min, obtaining the same yields. This result, together with those discussed below, confirm that an extraction of 45 min with ethanol-water (70:30 v/v) is sufficient. Therefore, these conditions were selected for all the following experiments.

3.2 Developing a new CE-UV method to analyze GEC

Due to the low stability of GEC at both acid and basic pHs, only volatile buffers with pH values around the physiological were tested. Namely, 20 mM ammonium hydrogen carbonate at pH 8, 50 mM ammonium hydrogen carbonate at pH 7.8, and 20 mM ammonium hydrogen carbonate at pH 7 were tested. Also, in order to optimize the CE-UV detection conditions, three different wavelengths were tested, namely, 200, 214, and 254 nm. The best S/N ratio for GEC was obtained using 200 nm under our separation conditions. Therefore, this value was used for all the subsequent experiments.

The best CE conditions consisted of using the 20 mM ammonium hydrogen carbonate buffer at pH 7, obtaining a fast separation of GEC in less than 4 min using a 37-cm capillary (see Fig. 1). The nature of this peak was initially confirmed by comparing the UV absorption ratios obtained from CE separations at different wavelengths with the theoretical ones from the UV spectrum of GEC [6]. Thus, the experimental ratios obtained at 254 nm/200 nm and 214 nm/200 nm were 0.21 and 0.73, respectively. These values are in good agreement with the theoretically expected, 0.20 and 0.79, respectively, which seems to indicate that the peak is due to GEC. This point was further corroborated by CE injecting a purified GEC sample (see Section 2) obtaining a peak at the same analysis time.

Figures 1A and B show the CE-UV electropherograms obtained after injecting the extracts obtained from a *V. narbonensis* seed flour using different ethanol percent-

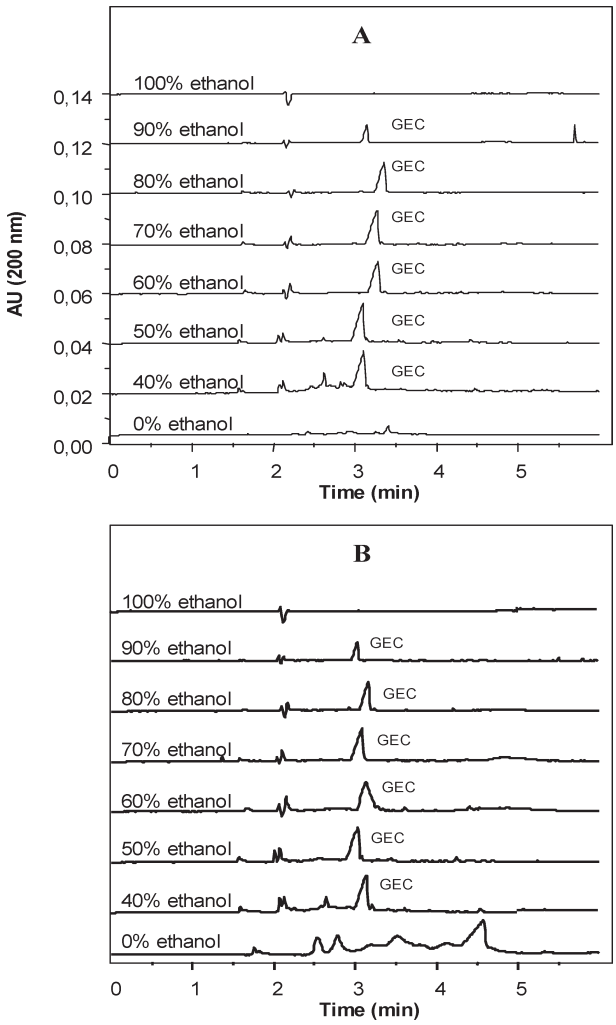

Figure 1. CE-UV electropherograms of GEC dipeptide extracted from Altair cultivar seed flour using different ethanol percentages. (A) 45 min extraction time, and (B) 24 h extraction time. CE conditions: Bare silica capillary (30 cm l_d, 37 cm l_t, 50 μm ID); BGE composition, 20 mM ammonium hydrogen carbonate at pH 7 (adjusted with formic acid). Running voltage, 15 kV (~17.5 μA); capillary temperature at 25°C; injection at 0.5 psi during 5 s.

ages after 45 min (Fig. 1A) and 24 h (Fig. 1B) of extraction. As can be seen, the use of ethanol percentages lower than 50% brings about the extraction of other non-desired compounds from the seed that comigrate with GEC or make more complex the CE-UV electropherogram. The use of 100% or 0% ethanol does not allow the extraction of GEC. The best GEC extractions were obtained by using 70% ethanol. Interestingly, by comparing Figs. 1A and B it can be deduced that the extraction yield is practically the same at 45 min and 24 h. In good agreement with these results, the same electropherograms were obtained after 2 h of extraction (data not shown).

3.3 CE-MS analysis of GEC

CE-ESI-MS application is limited by the use of volatile buffers [17–19] because the presence of nonvolatile components into the CE separation buffer decreases the sensitivity, increases the background noise and, under extreme conditions, can clog the system [20–22]. This problem has been solved in our work by selecting a volatile buffer composed of ammonium and carbonate. However, apart from the composition of the buffer, other factors have a large influence on the ESI-MS signal and, therefore, need to be optimized. Namely, sheath liquid composition, sheath liquid flow rate, and pressure of the nebulizer gas were optimized in this work.

In the first place, addition of acetic and formic acid to the sheath liquid composed by methanol-water (50:50 v/v) was evaluated. As can be observed in Fig. 2A, the addition of acid enhanced the S/N ratio in the extracted ion electropherogram (EIE) signal. Best response was observed for the sheath liquid containing 0.1% acetic acid. Later on, with a constant concentration of 0.1% acetic acid, the influence of methanol composition in the sheath liquid was studied at 0, 25, 50, 75, and 100%. As shown in Fig. 2B, higher sensitivity was obtained at 50% methanol composition. Also, the effect of flow rate of the sheath liquid (methanol-water 50:50 v/v containing 0.1% acetic acid) was studied over the range 1–6 μL/min (Fig. 2C). Slight improvement of S/N was achieved when 3 μL/min was used. The electrospray nebulizer pressure was next optimized using optimum sheath liquid composition and flow-rate (Fig. 2D). A tendency towards better sensitivities was observed when higher nebulizer pressures were used. In addition, lower migration times were obtained due to the suction effect produced by the nebulizer gas within the capillary column. Due to less stability of the signal when the nebulizer pressure was raised, a medium value of 11 psi was selected.

This new method was used for GEC analysis on several seed flour beans. Figure 3 shows an example of the CE-MS total ion electropherogram (TIE) and EIE obtained from an extract (using 45 min with ethanol-water (70:30 v/v)) and optimum CE-MS conditions. Moreover, the MS spectrum shown in Fig. 3 confirms that GEC is the main extracted compound for which a $[M+H]^+$ equal to 277.0 was obtained in good agreement with the theoretical value expected for this molecule (*i.e.,* 276.3 Da). In addition, four more peaks, numbered from 1 to 4 in Fig. 3A were detected with *m/z* values equal to 177.8, 174.0, 176.0 and 159.0, respectively. However, further investigation about the nature of these four compounds was discarded since this was out of the scope of this work.

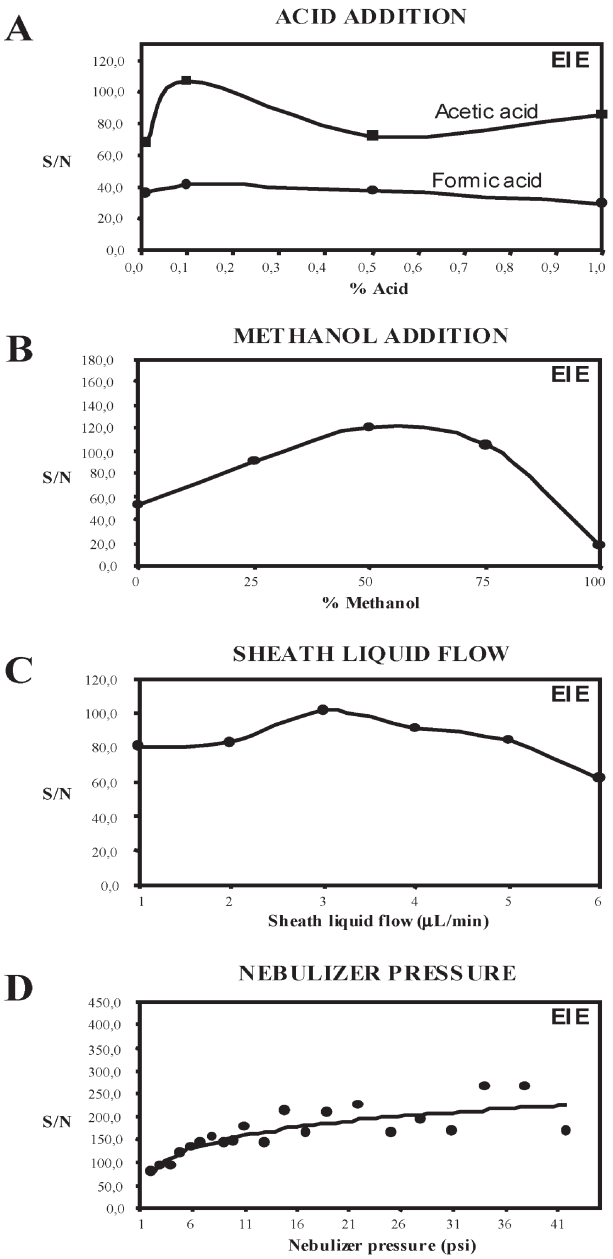

Figure 2. Electrospray optimization conditions and its influence on EIE S/N ratio. Effect of (A) acid addition to the sheath liquid, (B) methanol composition on the sheath liquid, (C) sheath liquid flow-rate, and (D) nebulizer pressure. Experiments were carried out using a dissolution of 1 mg/mL GEC.

In order to improve sensitivity of the GEC analysis, fragmentation of the 277 *m/z* parent ion was investigated under different MS/MS conditions; however, compared with that of the parent ion, no intensity improvement was obtained with the fragments and, therefore, single MS analysis of GEC was selected for the rest of the experiments.

3.4 CE-MS *vs.* CE-UV: a comparison between quantitative capabilities

Once optimum conditions were obtained for CE-UV and CE-MS analysis of GEC, a comparative study on the qualitative and quantitative capabilities of both procedures was carried out. Thus, reproducibility of GEC migration time was calculated for both methods (*i.e.,* CE-UV and CE-MS) for the same day and in three different days. It was observed that within the same day (n = 5) the %RSD values obtained were lower than 1.48% for CE-MS, and lower than 0.68% for CE-UV analysis of GEC. The inter-day study (3 days, n = 15) gave %RSD values lower than 1.9% for CE-UV and 2.3% CE-MS, showing comparable reproducibility. Next, the purified GEC sample was dissolved in ethanol-water (70:30 v/v) at different known concentrations from 0.98 to 4.9 mg/mL. In Fig. 4, calibration curves (injecting each concentration in triplicate) and residual plots from CE-UV and CE-MS methods are represented. Good correlation between signals from MS and UV detection and GEC concentration was observed. It can also be observed from the residual plots that there are not incompatibilities for a linear adjustment of the points. A comparison between the results from these two methods is given in (Table 2). As can be seen, CE-UV provides a faster analysis time than CE-MS as could be expected from the longer capillary needed for the later instrument (87 *vs.* 20 cm). Both procedures allow the quantitation in the range studied although the determination coefficient (r^2) for CE-UV is better (0.999) that from CE-MS (0.997). LOD, calculated for an S/N equal to 3 injecting a 0.025 mg/mL GEC dissolution (see Fig. 5), was similar for both techniques, although the LOD obtained with CE-UV (0.017 mg/mL) was slightly better than with CE-MS (0.021 mg/mL) for this compound. LOQ, calculated for an S/N ratio of 10, were equal to 0.057 and 0.071> mg/mL for CE-UV and CE-MS, respectively. The

Table 2. Performance parameters for CE-UV and CE-MS analysis of GEC

Parameter	CE-UV	CE-MS
Analysis time (min)	2.57	10.26
Concentration range (mg/mL)	0.98–4.9	0.98–4.9
Slope (b)	0.460	4396405
Intercept (a)	−0.011	464352
Determination coefficient (r^2)	0.999	0.997
RSD (%)[a)]	0.6–4.7	6.5–28.5
LOD[b)]	0.017	0.021

a) RSD (%), calculated from each triplicated injections (seven standard concentrations)
b) LOD (mg/mL), calculated for an S/N ratio equal to 3. Experimental conditions as given in Fig. 5.

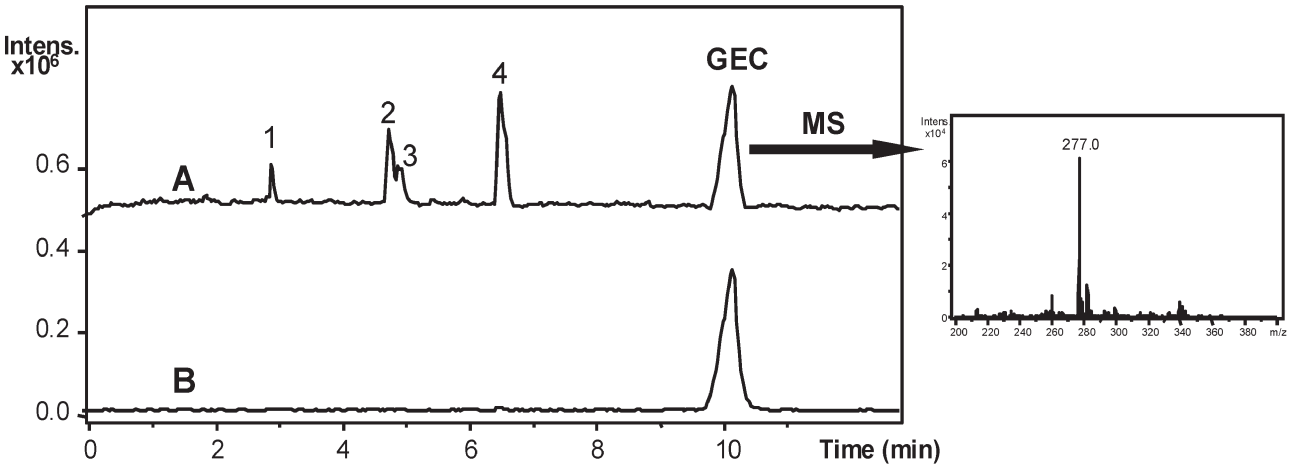

Figure 3. CE-MS (A) base peak electropherogram and (B) extracted ion electropherogram (277.0 *m/z*) of the BGE001894 extract. CE-MS conditions: Bare silica capillary (87 cm l_d, 87 cm l_t 50 μm ID). Running buffer composition: 20 mM ammonium hydrogen carbonate at pH 7, running voltage: 25 kV (~13 μA), capillary temperature at 25°C, injection at 0.5 psi for 12 s. MS positive ion mode, sheath liquid composition: methanol-water (1:1, v/v) with 0.1% acetic acid, at a flow rate of 3 μL/min, dry gas flow at 8 L/min. Temperature: 120°C. Mass scan: 200–400 *m/z*.

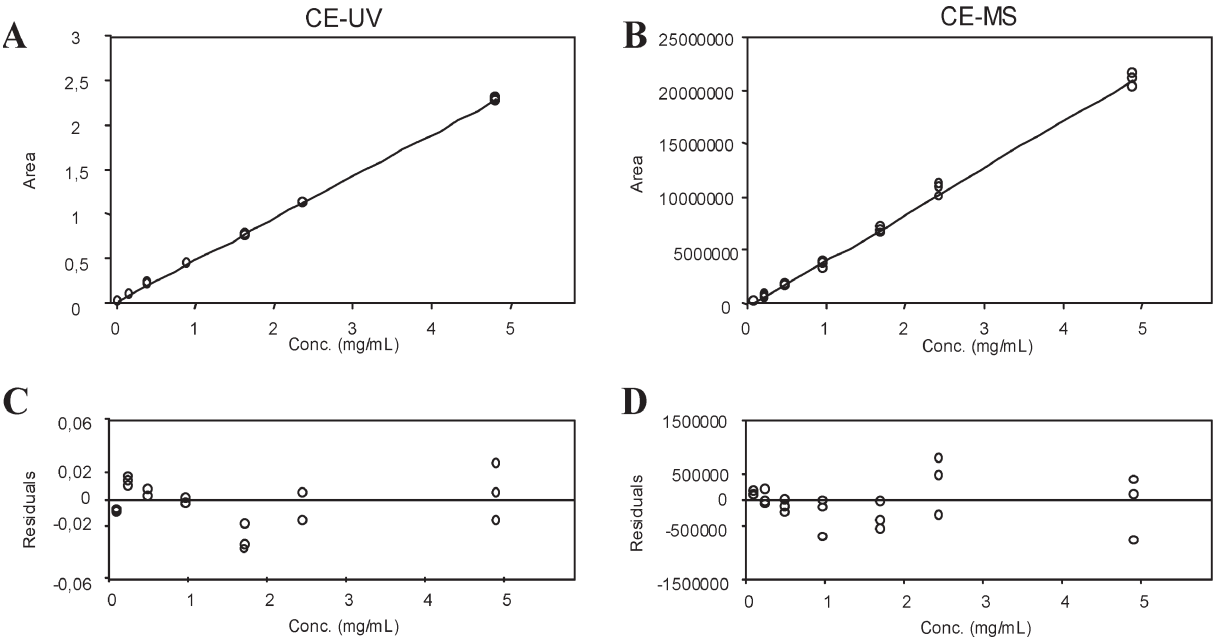

Figure 4. Calibration curves obtained for (A) CE-UV method and (B) CE-MS method. Residual plots corresponding to (C) CE-UV calibration and (D) CE-MS calibration.

higher sensitivity of the CE-UV method is due to the large UV absorption of the peptidic bond plus the high absortivity of the C-S group in GEC at the detection wavelength used (200 nm) [23]. Interestingly, the RSDs, calculated from the triplicated injections carried out at each calibration concentration, clearly show a better reproducibility of CE-UV than for CE-MS, since these values range from 0.6 to 4.7% using CE-UV, and between 6.5 and 28.5% using CE-MS.

Using the performance parameters shown in Table 2, it was possible to carry out the determination of the percentage of GEC in the 21 genotypes studied in this work. The results are given in the last column of Table 1. As can be seen, the content of GEC in the Narbon bean seeds analyzed is between 1.55 and 3.06%, in good agreement with the values reported in the literature for this species [2].

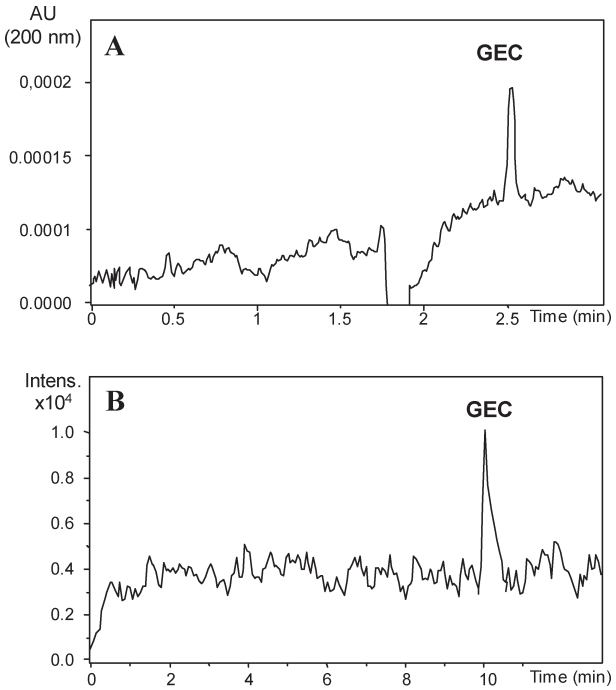

Figure 5. (A) CE-UV electropherogram and (B) CE-MS EIEs of GEC (277.0 *m/z*) from which LODs were calculated. GEC concentration: 0.025 mg/mL, injected at 0.5 psi for 12 s. CE-UV conditions: bare silica capillary (20 cm l_d, 87 cm l_t, 50 μm ID). CE-MS conditions as in Fig. 3.

Table 3. Comparison of the GEC (%) (dry weight) in different Narbon bean seed flour accessions determined by CE-UV and CE-MS (n = 2)

Sample No.	UV detection		MS detection	
	GEC (%)	RSD (%)	GEC (%)	RSD (%)
1	2.28	0.9	2.29	2.8
2	2.21	1.2	2.12	2.6
3	2.43	1.2	2.44	1.4
4	2.25	1.9	2.30	2.6
5	2.45	1.4	2.32	1.7
6	2.27	1.8	2.00	2.5
11	1.57	1.4	1.40	2.2
21[a)]	2.01	1.5	1.62	2.9

a) Commercial seed Altair (see Section 2.2, Samples)

Table 3 shows a comparison of the percent of GEC determined by CE-UV and CE-MS for a group of Narbon bean seeds arbitrarily chosen and using the calibration parameters given in Table 2. In order to compare the CE-UV and the CE-MS results of Table 3, a statistical comparison of the values provided by both methods was carried out using simple linear regression analysis. The regression obtained from the CE-UV and the CE-MS method was $\%GEC_{(by\ CE\text{-}MS)} = 1.227 \cdot \%GEC_{(by\ CE\text{-}UV)} - 0.616$, being $\%GEC_{(by\ CE\text{-}MS)}$ and $\%GEC_{(by\ CE\text{-}UV)}$ the values of Table 3 provided by the CE-MS and the CE-UV method, respectively. Moreover, the confidence limit for the slope was 0.720–1.733. Since the calculated confidence limit contains the value 1, it can be concluded that CE-MS provides similar results that CE-UV at the 95% confidence level. Therefore, a good agreement was obtained by both procedures demonstrating the accuracy of CE-UV and CE-MS methods developed in this work.

4 Concluding remarks

It can be deduced from our results that CE-UV is better suited for GEC analysis than CE-MS, mainly due to the low costs of the CE-UV instrument and better sensitivity obtained due to the large extinction coefficient of the solute at 200 nm. Besides, the quantitative analysis by CE-UV is more precise than by CE-MS. However, an evident advantage of CE-MS over CE-UV is that the detection of GEC could immediately have been done from the MS spectra, while availability of the purified GEC is mandatory for its CE-UV analysis.

CS thanks Consejería de Educación y Cultura (Comunidad de Madrid) for a fellowship. MA thanks INIA for a fellowship. This work was developed in the framework of the research project 197/CH-54, supported by Consejería de Agricultura of Castilla-La Mancha. The authors would also like to thank AGROSA, Semillas Selectas S.A. for supplying Altair seeds, Dr. Max E. Tate for the GEC standard, and Dr. Pedro J. Martín for his support in processing the statistical data.

Received September 28, 2004

5 References

[1] Bennett, S. J., Maxted, N., *Genet. Resour. Crop Ev.* 1997, *44*, 411–428.

[2] Castleman, G., *A Guide for Feeding Narbon Bean to Animals*, The State of Victoria. Department of Natural Resources and Environment, Australia 2000.

[3] Heazlewood, C., Bell, C., *Grower Guide*. *Tanami-Australia's First Commercial Narbon Bean,* The State of Victoria. Department of Natural Resources and Environment, Australia 2000.

[4] Korte, C. J., *Final Report. Development of Narbon Bean as a Commercial Crop for Low Rainfall Cropping Zones*, DAV 368. The State of Victoria. Department of Natural Resources and Environment, Australia 2000.

[5] Siddique, K. H. M., Loss, S. P., Regan, K. L., Jettner, R. L., *Aust. J. Agric. Res.* 1999, *50*, 375–387.

[6] Enneking, D., *The Toxicity of Vicia Species and their Utilisation as Grain Legumes*, Centre for Legumes in Mediterranean Agriculture (CLIMA) Occasional Publication Nº. 6, University of Western Australia, Nedlands W.A. 1955, pp. 52–77.

[7] López Bellido, L., in: Hernández Bermejo, J. E., León, J., (Eds.), *Neglected Crops: 1492 from a Different Perspective*, F.A.O. Publications, Rome 1994, pp. 273–288.

[8] Enneking, D., Delaere, I. M., Tate, M., *Phytochemistry* 1998, *48*, 643–645.

[9] Richardson, S. D., *Chem. Rev.* 2001, *101*, 211–254.

[10] Olivares, J. A., Nguyen, N. T., Yonker, C. R., Smith, R. D., *Anal. Chem.* 1987, *59*, 1230–1232.

[11] Brocke, A., Nicholson, G., Bayer, E., *Electrophoresis* 2001, *22*, 1251–1266.

[12] Tomer, K. B., *Chem. Rev.* 2001, *101*, 297–328.

[13] Schmitt-Kopplin, P., Frommberger, M., *Electrophoresis* 2003, *24*, 3837–3867.

[14] Soga, T., *Anal. Chem.* 2000, *72*, 1236–1241.

[15] Simó, C., López Soto-Yárritu, P., Cifuentes, A., *Electrophoresis* 2002, *23*, 2288–2295.

[16] Geiser, L., Rudaz, S., Veuthey, J.-L., *Electrophoresis* 2003, *24*, 3049–3056.

[17] Cai, J., Henion, J., *J. Chromatogr. A* 1995, *703*, 667–692.

[18] Huber, C. G., Premstaller, A., Kleindienst, G., *J. Chromatogr. A* 1999, *849*, 175–189.

[19] Gelpí, E., *J. Mass Spectrom.* 2002, *37*, 241–253.

[20] Ross, G. A., *LC-GC Europe* 2001, *1*, 2–6.

[21] Smith, R. D., Wahl, J. H., Goodlett, D. R., Hofstadler, S. A., *Anal. Chem.* 1993, *65*, 574–584.

[22] Niessen, W. M. A., Tjaden, U. R., Van der Greef, J., *J. Chromatogr.* 1993, *636*, 3–19.

[23] Pretsch, E., Buhlmann, P., Affolter, C., Herrera, A., Martinez, R., *Structure Determination of Organic Compounds*, Springer-Verlag, Berlin 2000.