DEVELOPMENT OF A NOVEL DUAL CD-MEKC SYSTEM FOR THE SYSTEMATIC FLAVONOID FINGERPRINTING OF *LIGARIA CUNEIFOLIA* (R. ET P.) TIEGH. – LORANTHACEAE – EXTRACTS.

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Abbreviations: S-β-CD (sulfated beta cyclodextrin); HP-β-CD (2-hydroxypropyl-β-cyclodextrin), DM-β-CD (Heptakis (2,6-di-O-methyl)-β-cyclodextrin), TM-β-CD (Heptakis (2,3,6-tri-O-benzoyl)-β-cyclodextrin); MeOH (methanol); Q (quercetin); Q-3-O-Rh (Quercetin-3-rhamnoside); R (rutin hydrate), PB2 (procyanidin B2), C (catechin), Q-3-O-G (quercetin-3-O-glucoside), Q-3-O-X (quercetin-3-O-xyloside), Q-3-O-AF (quercetin-3-O-arabinofuranoside); Q-3-O-AP (quercetin-3-O-arabinopyranoside), EAF (ethyl acetate fraction).

Keywords: CD-MEKC, fingerprinting, Flavonoids, *Ligaria cuneifolia*

ABSTRACT

The present work deals with the development and validation of a novel dual CD-MEKC system for the systematic flavonoid fingerprinting of *Ligaria cuneifolia* (R. et P.) Tiegh. -Loranthaceae- extracts. The BGE consisted of 20 mM pH 8.3 borate buffer, 50 mM SDS, a dual CD system based on the

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combination of 5 mM β-CD and 2% w/v S-β-CD, and 10% v/v methanol. The proposed method has been successfully applied to the comparative analysis of extracts from aerial parts and different hosts, geographical areas and extraction procedures in order to establish the flavonoid fingerprint of *L. cuneifolia*. The method was validated according to international guidelines. LOD and LOQ, intra and interday precision and linearity were determined for catechin, epicatechin, procyanidin B2, rutin, quercetin-3-O-glucoside, quercetin-3-O-xyloside, quercetin-3-O-rhamnoside, quercetin-3-O-arabinofuranoside, quercetin-3-O-arabinopyranoside and quercetin. The CD-MEKC methodology emerges as a suitable alternative to the traditional HPLC for quality control, fingerprinting and standardization of *L. cuneifolia* extracts from different sources.

1. INTRODUCTION

Ligaria cuneifolia (R. et P.) Tiegh. (Loranthaceae), the "Argentine mistletoe", is a widespread hemiparasitic plant in Argentina. It is employed in folk medicine as a substitute of the "European mistletoe", *Viscum album* L. (Santalaceae), because of its supposed action on decreasing high blood pressure. Polyphenols, and especially flavonoids, could be responsible for these actions [1].

Flavonoids are ubiquitous secondary plant metabolites that are widely used because of their spasmolytic, antiphlogistic, antiallergic, antioxidant and diuretic properties. Their common structure is that of diphenylpropanes (C6-C3-C6) and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (fig 1). They can be found as aglycones, but most commonly as glycoside derivatives. They are divided in different subgroups and they either occur as aglycones or as O- or C-glycosides. Among the flavonoids, flavones (e.g., apigenin, luteolin, diosmetin), flavonols (e.g., quercetin, myricetin, kaempferol), and their glycosides are the most common compounds [2]. Proanthocyanidins are oligomeric flavonoids composed of a flavan-3-ol or flavan-3,4-diol monomeric structure (such as catechin, epicatechin or gallocatechins). Previous studies on polyphenols in *Ligaria cuneifolia* reported the free or glycosilated quercetin as the only flavonol, together with leucocyanins and proanthocyanidins [1].

Traditionally, the determination of flavonoids has been performed by spectroscopic or chromatographic techniques (TLC, GC, HPLC). In this regard, HPLC has been the method of choice and hyphenated techniques (such as LC-DAD, LC-MS) where the separation step is coupled with an online spectroscopic detection have remarkably widened the applications to the analysis of complex matrices, especially natural products [3].

Capillary electrophoresis (CE) with its different modes of operation, has proven to provide great utility in the analysis of different types of compounds and to be an attractive alternative to traditional methodologies due to its high efficiency, reduced sample volume and reagent consumption, short analysis time, wide range of analysis and the possibility of making changes in the electrolyte composition in order to separate an ample span of hydrophobic and hydrophilic compounds in complex matrices [4]. CE has been applied to the separation of flavonoids since 1991 [5]. Borate buffers have been widely employed due to their ability to complex polyphenol aglycone and/or saccharides. Two hydroxyl groups of boric acid complex with cis-diol groups. Borate ions form charged and mobile five-membered-ring complexes (with 1,2-diols) and six-membered ring complexes (with 1,3-diols) and therefore, the separation selectivity is increased [6]. It is also confirmed by CE that an ion-dipole interaction takes place between flavonoids and borate [7]. The complex formation reaction is a strongly-pH dependent equilibrium which is favored at higher pH values [8]. Electrolyte additives have been used to modify the electrophoretic mobility of analytes for better separation. In particular, the development of MEKC, in which micelles are added to the electrolyte as pseudo-stationary phases, has greatly expanded the utility of the technique because it combines features of both LC and CE techniques. The combination of both separation mechanisms results in a powerful tool that makes possible to disentangle complex mixtures of analytes. The varying rates of partition between the complex analyte–micelle lead to excellent selectivity in separation [9, 10]. MEKC has also been used in combination with acidic buffers for the effective separation of proanthocyanidins in food samples [11].

The use of chiral stationary phases as well as chiral additives in HPLC is usually expensive since large amounts of stationary phases or acids are required. In CD-MEKC, several non-derivatized and derivatized CDs are used as chiral selectors and normally achiral ionic micelles as pseudostationary phases. The amount of CDs required is quite small and the costs of the enantioseparations by CD-MEKC will be considerably reduced compared to traditional HPLC [12].

Organic solvents can also be added to the electrolyte to affect the rate of inclusion complex formation; however, a high concentration of an organic solvent could reduce the number of micelles [9, 10].

Detailed reviews dealing with electromigration techniques for analysis of polyphenols (among other analytes) applied to food and foodomics are available in the literature [13 - 15]. The aim of this work was to develop a CE method for the systematic fingerprinting of *Ligaria cuneifolia* extracts. It was successfully applied to the analysis of specimens from different hosts and geographical regions of Argentina as well as various extraction procedures which makes it useful for extract standardization. To the best of our knowledge, this is the first report on the analysis of *Ligaria cuneifolia* extracts by a dual CD-MEKC system.

2. MATERIALS AND METHODS

2.1. Chemicals

Sodium tetraborate was from Carlo Erba (Rodano, MI, Italy). Sodium dodecyl sulfate (SDS), and cyclodextrins (CD): β-cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), Heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), Heptakis (2,3,6-tri-O-benzoyl)-β-cyclodextrin (TM-β-CD) and β-CD sodium sulfate salt (S-β-CD) were purchased from Sigma (St. Louis, MO, USA). Quercetin-3-rhamnoside (Q-3-O-Rh) was from Extrasynthese (Lyon, France) and rutin hydrate (R), procyanidin B2 (PB2), catechin (C), quercetin (Q), quercetin-3-O-glucoside (Q-3-O-G), quercetin-3-O-xyloside (Q-3-O-X), quercetin-3-O-arabinofuranoside (Q-3-O-AF) and quercetin-3-O-arabinopyranoside (Q-3-O-AP) were from Sigma (St. Louis, MO, USA). Methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF), ethyl acetate HPLC grade were supplied by Sintorgan (Argentina). Ultrapure water was obtained from an EASY pure™ RF equipment (Barnstead, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, USA) before use.

2.2. Plant material

L. cuneifolia (Ruiz & Pav.) Tiegh. (Loranthaceae) specimens were collected from different hosts and geographical areas. Aerial parts (stems, leaves and whole plant) were used. Plants were collected during October and November (post bloom) in the Argentinean northwest: in the province of San Juan (Barreal, Calingasta Department. Hosts: *Prosopis chilensis* (Molina) Stuntz, *Prosopis flexuosa* D.C and *Geoffroea decorticans* (Gillies ex Hook. & Arn.) Burkart) and for comparative purposes, in the province of Catamarca (Belen, Puerta de San José. Hosts: *Olea europea L.* -Oleaceae-, *Bulnesia retama* (Gillies ex Hook. & Arn.) Griseb. (Zygophyllaceae), *Geoffroea decorticans* (Gillies ex Hook. & Arn.) Burkart and *Prosopis flexuosa* D.C).

A commercial sample from *San Juan* (leaves) was also collected (Hosts: *Prosopis chilensis* (Molina) Stuntz, *Prosopis flexuosa* D.C and *Geoffroea decorticans* (Gillies ex Hook. & Arn.) Burkart).

Plants were taxonomically identified and voucher specimens were deposited in the herbarium of the *Museo de Farmacobotánica* for future references. Plant materials were air dried in the shade, ground to a fine powder and stored in closed flasks at -20^0 C in the dark until they were used.

2.3. Equipment

CE analysis was carried out with a P/ACE™ MDQ capillary electrophoresis system with diode array detector (Beckman, Fullerton, CA, USA). Uncoated fused silica capillaries (Microsolv technology, Eatontown, NJ, USA) of 60 cm (50 cm length to the detector) x 75 μm i.d. were used in all CE separations.

Ultrasonic bath (Transsonic Digitals, ELMA, Germany), mill (IKA, Germany), M23 orbital agitator (Vicking, Argentina) and a rotatory evaporator (Büchi, Germany) were used.

2.4. Extract preparation

Plant samples were reduced to a very fine powder (approx. 50 g) and successively extracted with hexane and dichloromethane for 24 hs and continuous shaking in order to eliminate chlorophylls and fats. The remaining material was submitted to successive extractions with methanol, and two combinations of methanol-water: (80:20) and (50:50). The organic solvent was reduced in a rotatory evaporator and the combined aqueous phases were collected and partitioned with ethyl acetate. The ethyl acetate fraction (EAF) was used for further studies.

A more exhaustive analysis was carried out on leaves, as they are the most used part for medicinal purposes. In this case, EAF were obtained from each methanol extraction conditions, resulting in four samples: combined MeOH EAF (cMeOH EAF); MeOH EAF (MeOH EAF); 80:20 MeOH:water EAF (80 MeOH EAF) and 50:50 MeOH:water EAF (50 MeOH EAF). Considering that an infusion is the usual processed form in which the plant is consumed by the general population, a 5 % w/v leaf infusion was prepared according to Farmacopea Argentina VII Ed [16, 17].

As for the samples from *Catamarca*, for comparative purposes, the concentrated methanol extract was employed.

2.5. Sample preparation

Stock standard solutions for each phenolic compound were prepared at 1 mg/mL in methanol.

San Juan samples: stock solutions of 10 mg/mL of the ethyl acetate fractions were diluted in methanol.

Catamarca samples: stock solutions of 20 mg/mL of the concentrated methanol extracts were diluted in methanol.

2.6. CE analysis

Before the first use, the capillary was conditioned by rinsing at 30 psi with 0.5 M KOH for 5 minutes, 0.1 M KOH for 5 minutes, water for 10 minutes and finally with the separation electrolyte for 10 minutes. The capillary was flushed between runs with 0.1 M KOH for 2 minutes, water for 2 minutes and with the separation electrolyte for 2 minutes.

The optimized BGE consisted of 20 mM pH 8.3 borate buffer, 50 mM SDS, 5 mM β-CD and 2 % w/v sulphated β-CD. Samples were injected under 0.3 psi for 3 s and electrophoretic system was operated under positive polarity at 20 kV. Sample vials storage and the separation were carried out at 25^{0}C. Diode array detection at 255 nm (for flavonol glycosides) and 280 nm (for proanthocyanidins) were used.

The samples were prepared as follows:

Mix standard solution: aliquots of each phenolic stock standard solution were combined and diluted with sample diluent to final concentration, as follows: C (12.0 μg/mL), EC (16.0 μg/mL), PB2 (16.0 μg/mL), R (8.0 μg/mL), Q-3-O-G (6,0 μg/mL), Q-3-O-X (4.6 μg/mL), Q-3-O-Rh (4.6 μg/mL), Q-3-O-AF (6.6 μg/mL), Q-3-O-AP (8.0 μg/mL), Q (8.0 μg/mL).

Sample solutions: aliquots of each sample (10 mg/mL of ethyl acetate fractions and 20 mg/mL of methanol extracts) were properly diluted with sample diluent to a final concentration of 0.4 mg/mL and 0.8 mg/mL, respectively.

2.7. CE validation

Validation was performed according to international guidelines [18]. The evaluated parameters were linearity, limit of detection (LOD), limit of quantification (LOQ) and precision (intra and interday). LOD and LOQ were calculated on the basis of signal-to-noise ratios of 3 and 10, respectively.

Linearity was tested in the range 50% - 300%, at seven levels (50%, 75%, 100%, 125%, 200% and 300%), each injected by triplicate (for concentrations range, see table 2).

Intraday precision was calculated as RSD for six replicates (n=6) and interday was calculated as RSD, for 3 days, six replicates each (n=18).

3. RESULTS AND DISCUSSION

3.1. CE methodology development

In order to achieve the optimal resolution for the 10 selected flavonoids, a joint strategy was employed, in which both the electrolyte composition and the operating conditions were evaluated. A borate buffer system was selected as a basis for further optimization. A summary of the studied variables is presented in table 1.

3.1.1. BGE optimization

Initially, a borate based CZE was developed in which concentration and pH were evaluated. Flavonoid pKa values are thought to vary between 7.3 and 12.5 because of the presence of phenyl hydroxyl groups [19]. Taking into account the acidic nature of the analytes under study (fig. 1), different combinations of borate concentration (20 to 30 mM) and pH (7.0-11.0) were evaluated. As flavonoids are weak acids, ionization of the hydroxyl moiety is achieved in a basic pH range. Borate complexation of flavonoids is also favored in alkaline conditions, so a pH range of 8 – 9 was selected. Higher pH led to extended running times with no further improvement. As for borate concentration, 20 mM was selected. Higher values led to prolonged running times, current increases and compromised resolution.

MEKC with SDS has also been proposed as an alternative to traditional CZE for flavonoid analysis [20, 21] where optimal resolution was not completely achieved. For ionic analytes, separation in MEKC is based on both the degree of ionization and the hydrophobicity. The use of SDS was tested at 50 mM as it is commonly employed [19, 20]. Thus, the optimum pH for the 20 mM borate buffer was set at 8.3. SDS improves the separation at pH 8.3 whereas it has less or no effect at a higher pH [20]. The separation selectivity of MEKC was better than that of CZE, because electrophoretic behavior in the latter is affected by more factors, such as the degree of saturation and the stereochemistry of the C-ring, alkyl substitution and the number and position of phenolic hydroxy groups, glycosylation of the hydroxy groups, as well as the complexation of flavonoids with a borate buffer [22]. Even though the addition of SDS resulted in a slight resolution improvement, complete separation was not fully accomplished. The migration order was as follows: C, EC, PB2, R, Q-3-O-G, Q-3-O-X, Q-3-O-Rh, Q-3-O-AF, Q-3-O-AP and Q. This is in accordance with literature since flavanols migrate first and then, flavonols. Cis/trans stereoisomerism at the C2/C3 carbons influences migration time in that the trans-isomers (catechin) migrate faster than the corresponding cis-isomers (epicatechin). Among flavonols, glycosylation of the hydroxyl groups also increases their hydrophilicity, leading to a decrease in migration time. Increasing the number of linked sugar molecules also reduces migration time even more [22]. For monosaccharides, migration times are in agreement with previous reports [8]. Thus, the migration order among flavonols was: diglycosides (rutin), monoglycosides (Q-3-O-G, Q-3-O-X, Q-3-O-Rh, Q-3-O-AF and Q-3-O-AP) and aglycone (quercetin). Three critical pairs were identified, with different structural features: catechin – epicatechin; Q-3-O-G -- Q-3-O-X and Q-3-O-Rh -- Q-3-O-AF. In the flavan-3-ol catechin-epicatechin pair, a chiral mechanism is involved. As for the flavonol glycosides, all of them are attached to the same aglycone moiety (quercetin) in the 3-O-position, so effective separation is based entirely on the ability to discriminate between sugar residues. All of these make successful separation especially challenging. Addition of a CD to the MEKC system gives rise to the inclusion-complexation equilibria of the analytes into the cyclodextrin cavity, which occurs simultaneously to the partitioning into the SDS micelle [23]. Moreover, solute-CD interactions are generally more selective than solute-micelle interactions. Solutes form inclusion complexes with CDs based on their size, geometry, and physicochemical properties, while

interactions with micelles are largely based on solute hydrophobicity [24]. CDs are effective chiral selectors and were explored as complementary additives to the MEKC system. Native neutral CDs such as β-CD and γ-CD and neutral CD derivatives as DM-β-CD, TM-β-CD, HP-β-CD were evaluated. β-CD at 5 mM led to the successful separation between catechin and epicatechin and a significant resolution improvement among the quercetin glycoside group. In this case, β-CD played a dual role by acting both as a chiral selector between epimers and a resolution promoter among quercetin glycosides. The rest of the evaluated neutral CDs, alone or in combination, were not as effective as β-CD. S-β-CD was also tested but resolution between catechin and epicatechin was compromised and Q-3-O-Rh and Q-3-O-F were not satisfactorily resolved. Considering that resolution was yet to be perfected among the quercetin glycoside pairs, a dual CD-MEKC system was pondered. In this sense, the use of a charged S-β-CD as a second CD was evaluated. Effective separation strategies in CE have been achieved with combinations of neutral and charged CDs as chiral selectors [25, 26]. This is because of the occurrence of additional ionic interactions that contribute to their chiral recognition. Charged CDs also increase the mobility difference between the free analyte and the drug–analyte complex. A charged CD, without enantioselectivity to a compound, can be used in the mixture because it will provide an apparent electrophoretic mobility to the analyte (and a subsequent resolution increase) while the neutral CD provides separation between the two enantiomers [27]. In this case, the combination of a neutral CD, such as β-CD, and the charged S-β-CD produced the separation between the remaining two critical pairs.

~~The use of organic modifiers is sometimes necessary to achieve proper separation of critical compounds.~~ The use of organic modifiers may be necessary to achieve proper analyte separation. For this purpose, methanol (5% – 40%) and acetonitrile (8 – 22%) are often employed [28]. They may increase the solubility of analytes, making CE analysis available for substances with slight solubility in pure aqueous buffer solutions. Organic solvents are favorably applied to enhance the separation selectivity of CE and the mobility of the electroosmotic flow [29]. Also, the elution window can be extended by adding certain organic solvents to the running buffer [24]. In this case, MeOH, ACN and THF were tested. Especially, 10% MeOH added to the dual CD-MEKC system drove to the final baseline separation needed in the quercetin glycoside cluster (fig 2).

3.1.2. Operating conditions optimization

The effect of the applied voltage was studied over the range 20–25 kV. 20 kV was found to provide the best results in terms of run-time and resolution between peaks. The effect of temperature on electrophoretic separation was examined over the range 20–25°C. A temperature of 25°C was selected as optimal because it provided the best compromise between migration time and peak resolution. Pressure application during the run (0.1 psi) was also evaluated but no significant improvement was achieved.

The injection mode giving the best response concerning precision and linear range was the hydrodynamic mode. Injection parameters were optimized by varying the lengths of sample (2–5 s) and pressure injection until optimal conditions were reached. The best results were obtained with the following experimental parameters: hydrodynamic injection mode 0.3 psi, 3 s.

3.1.3. Sample diluent optimization

Several variables were considered for optimization: sodium tetraborate concentration and pH as well as additives. The inclusion of S-β-CD in the sample matrix was also evaluated as it was a good

component for providing a pronounced stacking effect and subsequent enhancement in sensitivity for detecting hydrophobic compound as well as being an excellent solubilizing agent [30]. When the sample was prepared in a low conductivity diluent, a stacking mechanism took place, explained on the basis of field amplification [31]. Hence, the best results in terms of peak symmetry and efficiency were obtained with 2 mM pH 8.3 sodium tetraborate buffer containing 20% MeOH.

The corresponding electropherogram of the mix standard solution at the optimal condition is depicted in Figure 2. The electropherograms were monitored at 255 nm (for flavonol glycosides) and 280 nm (for proanthocyanidins).

3.2. CE validation

Validation was performed according to international guidelines [18]. The evaluated parameters were limit of detection (LOD), limit of quantification (LOQ), linearity and precision (intra and interday). For flavonol glycosides and quercetin, the selected wavelength was 255 nm; 280 nm was chosen for flavan-3-ols. A summary of the results is listed in table 2.

LOD and LOQ were calculated at signal-to-noise ratios of 3 and 10, respectively. LOD values varied between 0.08 µg/mL to 0.40 µg/mL and LOQ, between 0.26 µg/mL to 1.33 µg/mL for Q-3-O-AF and EC, respectively.

Linearity was tested in the range 50% - 300%, with six concentration levels (50%, 75%, 100%, 125%, 200% and 300%), each injected by triplicate (for concentrations range, see table 2) . The correlation coefficient varied between 0.9923 and 0.9978.

Intraday precision was calculated as RSD for six replicates (n=6) and interday was calculated as RSD, for 3 days, six replicates each (n=18). The intraday RSD of the migration time was between 0.2 and 0.6 and for corrected area (peak area/tm) was between 1.6 % and 2.8 %. The interday values for the same performance criteria were 0.30 % – 0.7 % and 2.10 % – 3.20 %, respectively.

3.3. CE application to *L. cuneifolia* extracts

The validated methodology was applied to the analysis of *L. cuneifolia* extracts in order to produce flavonoid fingerprints for an effective quality control.

Specimen provenance, hosts, used part and extraction procedures were compared.

Representative electropherograms of ethyl acetate fractions from *San Juan* are shown in Fig. 3 A. Ten analytes were identified by means of co chromatography, isolation and purification, mass spectrometry and NMR: C, Q-3-O-G, Q-3-O-X, Q-3-O-Rh, Q-3-O-AF, Q-3-O-AP, previously described in *L. cuneifolia* [1]. Four novel galloyl quercetin glycoside derivatives were reported for the first time in *L. cuneifolia*. Galloylation led to prolonged running times with respect to their glycosides counterparts. The migration order is similar to the glycoside cluster with rhamnosides migrating faster (for which two positional isomers were identified), then arabinofuranosides and arabinopyranosides. Hence, the order among galloyl derivatives was as follows: quercetin-3-O- (2''-O-galloyl) rhamnoside, quercetin-3-O- (3''-O-galloyl) rhamnoside, quercetin-3-O- (2''galloyl)-arabinofuranoside and quercetin-3-O-(2''-O-galloyl)-arabinopyranoside. Comparing aerial parts (leaves and stems), in general, there are no quality differences and only minor quantitative ones. The same consideration applies to extraction procedures on leaves, which exhibit similar profiles.

Representative electropherograms of concentrated methanolic extracts from *Catamarca* and the commercial sample from *San Juan* are shown in Fig. 3 B. Comparing the same hosts (*Prosopis chilensis*, *Prosopis flexuosa* and *Geoffrea decorticans*, which belong to the Fabaceae family), similar

profiles were obtained for the ethyl acetate fractions from *San Juan* and the concentrated methanolic extracts from *Catamarca* but quantitative differences were observed. This is expected as the ethyl acetate partition generates enriched flavonoid fractions. The aqueous extract (infusion, the usual way in which *L. cuneifolia* is consumed), obtained from the commercial sample of *San Juan* produced a similar profile, with lower quantities of each analyte (data not shown).

4. CONCLUSIONS

The developed CE methodology has proven to be suitable for flavonoid fingerprinting and can be successfully applied to the quality control of *L. cuneifolia* extracts. Ten analytes have been resolved in a complex matrix within 25 minutes. Four novel compounds were reported for the first time in *L. cuneifolia*. The method has been validated according to international guidelines with satisfactory results. In contrast with findings from other authors, we did not find quercetin diglycosides (such as rutin) or kaempferol derivatives among the evaluated specimens [32].

The novel CE methodology also presents several advantages over traditional approaches: improved resolution, high number of theoretical plates, short running times, low cost and an eco- friendly alternatively for its low solvent consumption, just to name a few. For all the abovementioned, CE positions as the “green” choice for plant extract standardization.

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Figure 1: structure of common flavonoids.

R=H (A, quercetin)

R= glucose (B), xylose (C), arabinopyranose (D), arabinofuranose (E), rhamnose (F)

R1= gallic acid (G)

Catechin (H), epicatechin (I), Procyanidin B2 (PB2, J)

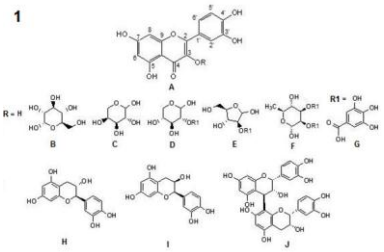

Figure 2: Mix standard solution

A: 255 nm

B: 280 nm

1 (C); 2 (EC); 3 (R), 4 (PB2); 5 (Q-3-O-G), 6 (Q-3-O-X), 7 (Q-3-O-Rh), 8 (Q-3-O-AF), 9 (Q-3-O-AP), 10 (Q)

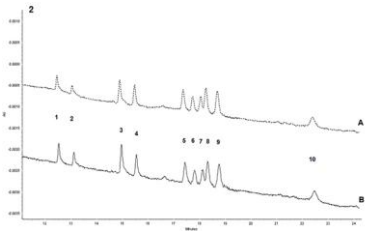

Figure 3: CE fingerprints of samples from *San Juan* and *Catamarca*

A: EAF from *San Juan* at 255 nm

a-d (leaf): a (combined methanolic extract); b (methanolic extract); c (80% methanolic extract); d (50% methanolic extract)

e (stem, combined methanolic extract)

B: methanolic extracts from *Catamarca* and commercial sample from *San Juan* at 255 nm

a (San Juan, commercial sample)

b-e (samples from *Catamarca* on different hosts): b (*Geoffroea decorticans*); c (*Olea europaea*); d (*Bulnesia retama*), e (*Prosopis chilensis* and *Prosopis flexuosa*)

1 (C), 2 (Q-3-O-G), 3 (Q-3-O-X), 4 (Q-3-O-Rh), 5 (Q-3-O-AF), 6 (Q-3-O-AP), 7 (Q-3-O-(2''-galloyl) rhamnoside), 8 (Q-3-O-(3''-galloyl) rhamnoside), 9 (Q-3-O-(2''-galloyl) arabinofuranoside), 10 (Q-3-O-(2''-galloyl) arabinopyranoside).

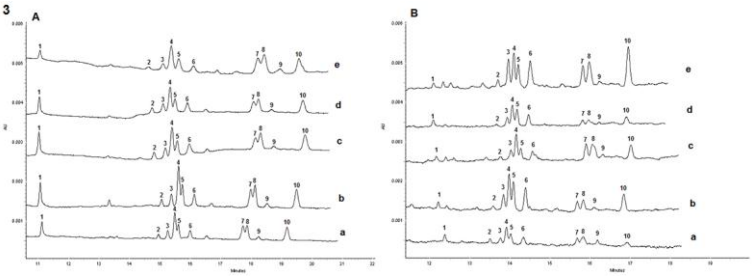

Table 1: optimization of the electrolyte composition

Borate		Additives										
		SDS	CD's						Organic solvents			
			β	HP-β	DM-β	TM-β	S-β	γ	MeOH	ACN	THF
(mM)	pH	(mM)	(mM)	(mM)	(mM)	(mM)	(%w/v)	(mM)	(%)	(%)	(%)
20-25-30	7.0	---	---	---	---	---	---	---	---	---	---
20-25-30	8.0	---	---	---	---	---	---	---	---	---	---
20-25-30	9.0	---	---	---	---	---	---	---	---	---	---
20-25-30	10.0	---	---	---	---	---	---	---	---	---	---
20-25-30	11.0	---	---	---	---	---	---	---	---	---	---
20	8.3	50	---	---	---	---	---	---	---	---	---

20	8.3	50	2.5-5-10	---	---	---	---	---	---	---	---
20	8.3	50	---	2.5-5-10	---	---	---	---	---	---	---
20	8.3	50	---	---	2.5-5-10	---	---	---	---	---	---
20	8.3	50	---	---	---	2.5-5-10	---	---	---	---	---
20	8.3	50	---	---	---	---	1.5-2-2.5	---	---	---	---
20	8.3	50	---	---	---	---	---	2.5-5-10	---	---	---
20	8.3	50	5	5	---	---	---	---	---	---	---
20	8.3	50	5	---	5		---	---	---	---	---
20	8.3	50	5	---	---	5	---	---	---	---	---
20	8.3	50	5	---	---	---	---	5	---	---	---
20	8.3	50	5	---	---	---	1.5- 2.0	---	---	---	---
20	8.3	50	5	---	---	---	2.0	---	5 - 10	---	---
20	8.3	50	5	---	---	---	2.0	---	---	5-10-15	---
20	8.3	50	5	---	---	---	2.0	---	---	---	2.5-5
20	8.3	50	5	---	---	---	2.0	---	5	5	---
20	8.3	50	5	---	---	---	2.0	---	10	10	---
20	8.3	50	5	---	---	---	2.0	---	5	15	---
20	8.3	50	5	---	---	---	2.0	---	10	15	---
20	8.3	50	5	---	---	---	2.0	---	10	5	---
20	8.3	50	5	---	---	---	2.0	---	5	10	---
20	8.3	50	5	---	---	---	2.0	---	---	15	2.5
20	8.3	50	5	---	---	---	2.0	---	---	15	5
20	8.3	50	5	---	---	---	2.0	---	10	---	2.5
20	8.3	50	5	---	---	---	2.0	---	10	---	5

Table 2: Validation parameters of the CE developed method

Analyte	LOD (µg/mL)	LOQ (µg/mL)	Linearity			Precision			
						RSD			
						Intraday (n=6)		Interday (n=18)	
			Regression equation *Y (AU) = a(AU/µg/mL) x (µg/mL) + b (AU)*	Correlation coefficient *(R)*	Range 50 % - 300% (µg/mL)	Corrected Area (AU/tm)	tm (min)	Corrected Area (AU/tm)	tm (min)
C	0.27	0.90	174x + 9.64	0.9939	6.0 – 36.0	1.6	0.3	2.1	0.5
EC	0.40	1.33	71x – 2.89	0.9926	8.0 – 48.0	2.0	0.3	2.5	0.4
PB2	0.33	1.11	182x + 10.4	0.9940	8.0 –	2.5	0.2	3.0	0.3

					48.0				
R	0.14	0.47	348x + 5.77	0.9932	4.0 – 24.0	2.7	0.3	3.1	0.5
Q-3-O-G	0.11	0.35	448x + 0.88	0.9936	3.0 – 18.1	2.6	0.4	3.2	0.5
Q-3-O-X	0.11	0.36	485x – 3.79	0.9923	2.3 – 13.7	3.0	0.4	3.2	0.6
Q-3-O-Rh	0.09	0.30	595x – 1.10	0.9924	2.3 – 13.8	3.1	0.4	3.2	0.5
Q-3-O-AF	0.08	0.26	625x + 7.36	0.9944	3.3 – 19.7	1.7	0.4	2.5	0.5
Q-3-O-AP	0.09	0.31	545x + 7.97	0.9978	6.0 – 36.0	2.8	0.5	3.2	0.6
Q	0.10	0.33	567x – 7.40	0.9972	8.0 – 48.0	2.7	0.6	3.2	0.7