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Characterization of carboxylate-terminated carbosilane dendrimers and their evaluation as nanoadditives in capillary electrophoresis for vegetable protein profiling

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ABSTRACT

Protein profiles are becoming an important tool to differentiate and classify varieties of several cultivars and to obtain a specific fingerprint for them. The use of protein profiles for these purposes needs to achieve high separation efficiencies to obtain a high number of well resolved peaks. In this work, carbosilane dendrimers with interior carbon–silicon bonds and negatively charged in the dendrimer surface with carboxylic acid as functional groups were employed as nanoadditives to separate soybean and olive seeds proteins. First, these dendrimers were characterized using CE to evaluate their possible impurities. A potentiometric titration was later carried out to determine their pK_a values. Afterwards, the characterized dendrimers were used to improve the protein profiles obtained by EKC for vegetable proteins. Different dendrimer generations (G1, G2, and G3) and concentrations (0.01–1% m/v) were tested. The highest dendrimer generation G3 at 0.1% (m/v) allowed observing the best protein profiles for soybean and olive seeds. These results demonstrate that carboxylate-terminated carbosilane dendrimers are attractive nanoadditives in EKC for the effective separation of vegetable proteins.

1. Introduction

Capillary electrophoresis is a powerful analytical tool for the separation of macromolecules with numerous applications such as the analysis of proteins in the food field. The works devoted to the analysis of food proteins by CE have mainly been focused on the quantitative analysis of the most important nutritional proteins in different dairy products or on the evaluation of the quality and authenticity of food products [1,2]. Moreover, protein analysis by CE has enabled the detection of food adulterations and the identification of different cultivars or varieties [1,2].

EKC is a separation mode very suitable for the analysis of proteins [3]. In this CE mode, analytes are separated based on their relative affinity for a pseudostationary phase [4]. The most widely employed pseudostationary phases in EKC for protein separations are conventional micelles from surfactants, such as SDS, or cyclodextrins, which interact with proteins facilitating their separation. Another option to achieve electrokinetic separations is the use of soluble polymer ions migrating counterflow to EOF. An example is the employ of dendrimers, symmetrical macromolecules with three-dimensional structures. Although the characterization of new dendrimers or some modifications of their structures by CE [5–7], as well as the use of dendrimers to coat some capillaries [8], have been reported, the use of dendrimers as pseudostationary phases in EKC has poorly been studied. Some of these uses were reviewed by Palmer et al. [4] and Castagnola et al. [6]. Dendrimers can act improving some separations of diverse compounds in CE [6], such as different amino acids [9], benzene derivatives [10], alkyl phenyl ketones [11], parabens [12,13], isomers of dimethyl phenol [14], and some aromatic compounds [15]. In most of these applications, better separation and higher resolution were generally observed using dendrimers in comparison with classical methods as SDS-MEKC. One of the major advantages of dendrimers is their potential to be used in systems in which a high proportion of organic solvents is required for the separation of compounds of

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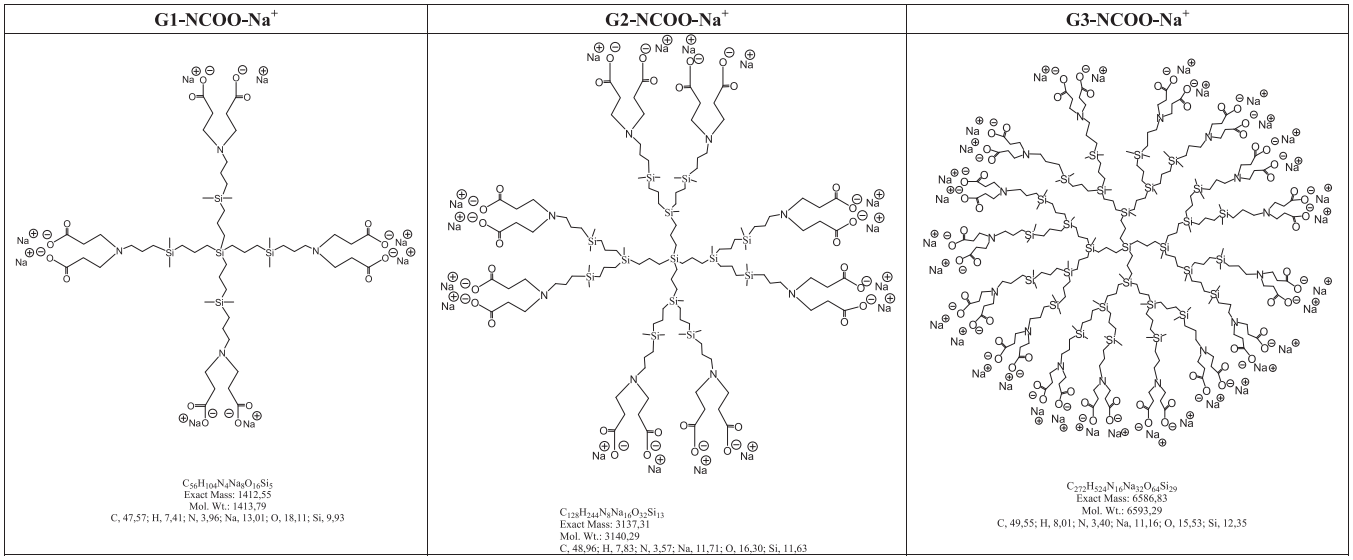

Fig. 1. Structures of the first (G1), second (G2), and third (G3) generations of the carboxylate-terminated carbosilane dendrimers used in this work. Molecular formula, molecular weight, and composition are also indicated.

hydrophobic nature, not possible in SDS systems. Another important benefit of using dendrimers in the separation buffer in CE is based on their uniform and versatile structure. Indeed, skeletons and surfaces of dendrimers may be modified changing parameters such as concentration, structure, generation, and their cationic or anionic nature [6] to allow a wide range of applications and to improve the separation selectivity.

Dendrimers interactions with proteins have also been investigated originating their use in some important applications. As example, some dendritic systems can act as nanocontainers of drugs and they can release them in response to protein–dendrimer interaction [16]. The interactions depend on the peripherical groups of dendrimers and the pH of the medium. Thus, the interaction between carbosilane dendrimers, with peripherical ammonium or amine groups, and a standard protein (bovine serum albumin, BSA) were tested by Pedziwiatr et al. [17] using fluorescence quenchers. Conformational changes were not found in the protein due to the interaction with dendrimers. The results showed that interactions between these dendrimers and BSA were weak and occurred preferentially at the protein surface [17]. Employing circular dichroism, Shcharbin et al. [18] also established that dendrimers did not significantly affect the protein secondary structure. Structural analysis showed that dendrimers bind BSA via hydrophilic and hydrophobic interactions, carrying out the analysis using poly(amidoamine) (PAMAM) dendrimers [19]. These electrostatic and non-electrostatic interactions can give rise to stable complexes [20]. Thus, due to their mode of interaction with proteins, which involves ion-exchange interactions and hydrophobic partition, the dendrimers can be applied to the separation of proteins [21,22]. Moreover, dendrimers can act as strong ion pairing or competing agents for the basic amino acid residues of the proteins and subtract their availability to the silanol groups on the capillary wall, avoiding the adsorption in the capillary wall. Although the information described above highlights that dendrimers may be a good alternative to improve protein separation in CE, this fact has only been investigated in two works [21,22]. In the first case, cationic and anionic Starburst PAMAM dendrimers of different generations were used to improve profiles of chicken sarcoplasmic proteins [21]. The other work examined the influence of cationic PAMAM dendrimers on the separation of some standard proteins by CE [22].

Therefore, it seems very interesting and promising to study new water soluble dendrimers in order to investigate their effect on the separation of proteins by EKC and the improvement in protein profiles. These profiles are useful tools for the differentiation and classification of varieties of several foods and to obtain their specific fingerprinting. The usefulness of proteins profiles mainly depends on the reproducibility of the electrophoretic method and the number of well resolved peaks obtained, to allow the comparison of several protein profiles and to assure the specificity of them. In this article, carbosilane dendrimers with interior carbon–silicon bonds and carboxylic acid as functional groups in the dendrimer surface, showing a negatively charged outer layer, were firstly characterized using CE and potentiometry titration and employed later as pseudostationary phase in EKC. Then, these anionic carbosilane dendrimers were evaluated as nanoadditives in EKC to obtain improved protein profiles for vegetable proteins.

2. Materials and methods

2.1. Reagents and materials

HPLC grade ACN, chloroform, acetone, tetrahydrofuran (THF), isopropanol, methyl ethyl ketone, and dithiothreitol (DTT) were obtained from Scharlau Chemie (Barcelona, Spain). Urea, hydrochloric acid, boric acid, Tris (tris(hydroxymethyl)aminomethane), SDS, and sodium hydroxide pellets were purchased from Merck (Darmstadt, Germany), dimethyl sulfoxide (DMSO) from Fluka (Buchs, Switzerland), and guanidine from Sigma (St. Louis, MO). All solutions were prepared with ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA). All buffers were filtered prior to use through a 0.45 μm pore size disposable nylon filters from Titan (Titan 2, Eatontown, NJ). Soybean protein isolate (SPI) used during the method development was from ICN Biomedicals (Aurora, OH, USA). The olive samples analyzed in this work belonged to Hojiblanca variety and were collected in Toledo (Spain).

Carboxilated carbosilane dendrimers of first (G1), second (G2) and third (G3) generation were synthesized by reaction of amine functionalized dendrimers $G_nSi(NH_2)_m$ ($n=1$, $m=4$; $n=2$, $m=8$; $n=3$, $m=16$) with methyl acrilate, $C_2H_3CO_2Me$, through a Michael-type addition and subsequent treatment with sodium hydroxide

(NaOH), [23] and were used without further purification. In Fig. 1, the structures of the different dendrimers used are shown. As it can be seen, the number of terminal groups of dendrimers and the molecular mass rapidly increase with the generation. These dendrimers have 8, 16, and 32 surface carboxylate groups, respectively, and molecular masses ranged from 1413.79 Da to 6593.29 Da. Dendrimer solutions were prepared in a borate buffer (100 mM, pH 9.0) and used at concentrations between 0.01% (m/v) and 1% (m/v).

2.2. Sample preparation

Soybean proteins were extracted using a mixture of water:ACN (80:20, v/v) by sonication during 3 min and centrifugation (Heraeus Instrument, Hanau, Germany) at 4000 × *g* for 3 min [24].

Protein extracts of olive samples were prepared from olive seeds from Hojiblanca variety according to a previous procedure developed by Wang et al. [25], with some minor modifications. Briefly, the olive fruits were depulped and the stones were cut to collect the olive seeds, being the seeds homogenized using a domestic miller (Kenwood Ibérica, Barcelona, Spain). Proteins from 500 mg of olive seeds were extracted with 5 mL of 125 mM Tris–HCl (pH 7.5), 1% (m/v) SDS, and 0.5% (m/v) DTT, previously optimized in our research group for olive stone [26], and it was vortexed vigorously during 2 min. A centrifugation at 4000 × *g* for 15 min was carried out three times. After centrifugations, 5 mL chloroform were added to the liquid phase and the two phases were separated by centrifugation at 4000 × *g* for 15 min. Proteins in the organic phase were precipitated with 2 volumes of cold acetone at −20 °C for 30 min. Precipitated proteins were separated by centrifugation 4000 × *g* for 15 min, and washed with 90% (v/v) acetone. Proteins were solubilized in 0.5 mL of 100 mM borate buffer (pH 9) and filtered through 0.45 μm nylon filters prior to their injection in the CE system.

2.3. CE procedure

A HP^{3D}CE instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with an on-column DAD for UV detection and spectra collection was used. All experiments were performed in fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm id and effective length of 50 cm (58.5 cm of total length). A capillary conditioning between sample injections with 0.1 M sodium hydroxide (1 bar) for 4 min, Milli-Q water (1 bar) for 4 min, and separation buffer (1 bar) for 5 min was achieved. When carbosilane dendrimers were used in the separation buffer, conditioning between samples was as followed: 1 M sodium hydroxide (1 bar) for 4 min, followed by Milli-Q water (1 bar) for 4 min, and separation buffer with dendrimers for 4 min (1 bar). The CE selected conditions were: capillary temperature 20 °C, applied voltage 15 kV, and UV detection at 254 nm with a bandwidth of 5 nm. The sample injection was performed by pressure, 50 mbar for 5 s for isolate soybean proteins and 50 mbar for 10 s for olive seed proteins. A 100 mM borate buffer (pH 9) without and with carbosilane dendrimers was used as separation buffers.

2.4. Potentiometric titration

Titration of carbosilane dendrimers was carried out using an acid–base potentiometric titration with a pHmeter Dismadel (Madrid, Spain). As titrator, different solutions of HCl 0.0105 and 0.0102 M were prepared, previously titrated in triplicate with 1 mM Na_2CO_3.

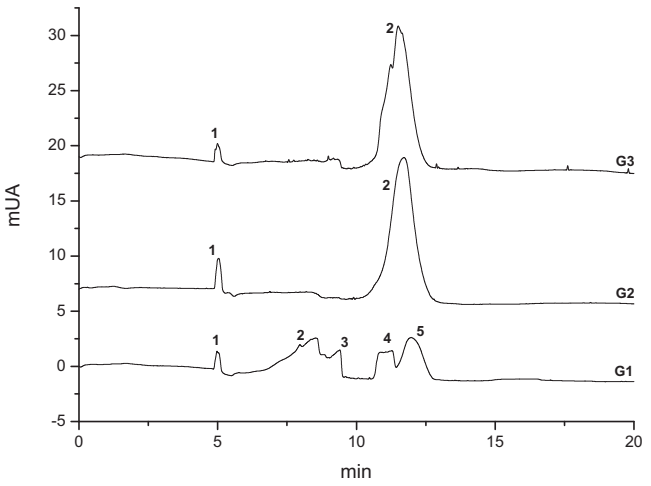

Fig. 2. CZE separations of carboxylate-terminated carbosilane dendrimers of generations 1, 2, and 3 at 0.5% (m/v) in 100 mM borate buffer at pH 9.0. The electrophoretic conditions were: 100 mM borate buffer (pH 9.0); capillary of 75 μm id and 50 cm effective length (58.5 cm total length); hydrodynamic injection, 50 mbar for 10 s; capillary temperature, 20 °C; applied voltage, 15 kV; UV detection at 214 nm (5 nm bandwidth).

3. Results and discussion

3.1. Characterization of carbosilane dendrimers by CE

It is known that many questions concerning the differences between theoretical and experimental dendrimer properties occurred. It is thus important to have analytical tools capable of reliable dendrimer characterization, evaluating the purity of dendrimers and studying different properties of dendrimers. CE was employed in this work as an efficient analytical technique allowing dendrimer characterization. With this purpose, three different generations of the carboxylate-terminated carbosilane dendrimers studied in this work were characterized.

Dendrimers solutions were prepared by dissolving them in the separation buffer (100 mM borate buffer pH 9.0) at a concentration of 0.5% (m/v). Each dendrimer generation solution was injected at 50 mbar during 10 s, employing the same electrophoretic conditions described in the CE Procedure section except the UV detection, fixed in this case at 214 nm with a bandwidth of 5 nm. Fig. 2 shows the CE separations at pH 9.0 of carboxylate-terminated carbosilane dendrimers of 1, 2, and 3 generations at 0.5% (m/v). It is known that dendrimers can give imperfect structures depending on monomer purity and reaction efficiency [27] and their syntheses, particularly by divergent protocols, often results in imperfect or impure structures. As Fig. 2 shows, all electropherograms exhibited one initial peak at 5.0 min, which corresponded to the electroosmotic flow. G1 presented another four peaks, G2 only showed other broad peak, and G3 showed one peak with a shoulder. Results obtained agreed with the fact that the separation of the impurities becomes more difficult for the highest generations [27]. According to Shi et al. [28], this could be attributed to a decrease in the charge/mass ratio differences among higher generations and their imperfect structures.

3.2. Acid–base behavior of the carboxylate-terminated carbosilane dendrimers

An acid–base titration by potentiometry has been carried out using the three anionic carboxylate-terminated carbosilane dendrimers studied in this work. In the titration curves obtained, two equivalence points were observed for the three generations of dendrimers. The first derivative allowed the determination of the exact

Table 1
pK_a values of carboxylated-terminated carbosilane dendrimers.

Gn-[$CH_2CH_2CH_2N(CH_2CH_2COO^-Na^+)_2]_m$						
Amino group				*Carboxyl group*		
	pKa	**Ka**			**pKa**	**Ka**
G1	10.4	$10^{-10.4}$	↓ Basicity	↑ Acidity	5	10^{-5}
G2	10.7	$10^{-10.7}$			5.64	$10^{-5.6}$
G3	11.2	$10^{-11.2}$			6.6	$10^{-6.6}$

value of these two points. The first point corresponded to the protonation of the amino group and the second one corresponded to the protonation of the carboxyl group (see Fig. 1 for structural data of each dendrimer).

pK_a was calculated using the Henderson–Hasselbalch equation. The results for the amino and carboxyl groups are collected in Table 1. As it can be seen, a dendritic effect exists in the basicity of amino group due to the fact that when the dendrimer generation increases the basicity of the amino group increases. For the carboxylic group, the acidity of this group decreases with the dendrimer generation. The results obtained were in agreement with the pK_a calculated by van Duijvenbode et al. [29] for dendrimers poly(propylene imine) (PPI) with carboxylic surface groups of 10.4 for the amino group and 4.40 for the carboxylic group. The estimation of the pK_a of each dendrimer can allow defining their pH-dependent characteristics and to know the species existent in physiological pH conditions for further applications.

3.3. Effect of carboxylate-terminated carbosilane dendrimers on the separation of soybean proteins

A SPI was selected as reference vegetable proteins for the optimization of the electrophoretic separation because contains 89.1% (m/m) (determined by Kjeldahl method) of proteins [30] and there are not standard proteins from olives. First, the protein profile for the soybean proteins without the addition of dendrimers was studied using basic separation conditions previously reported for soybean proteins [30]. In our case, these experimental conditions were: capillary of 75 μm id and 50 cm effective length (58.5 cm total length); hydrodynamic injection, 50 mbar for 5 s; capillary temperature, 20 °C; applied voltage, 15 kV; UV detection at 254 nm (5 nm bandwidth), and a separation buffer of 100 mM borate at pH 9.0. The sample concentration was 50 mg/mL. Under these conditions, the electropherogram showed a single wide peak (result not shown). Then, different sample concentrations for the isolate soybean proteins were tested: 100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL, choosing 50 mg/mL because provided good signal intensity. To improve the proteins separation of a SPI solution at 50 mg/mL, some experimental conditions were optimized prior to the addition of dendrimers. Thus, different pH values and different buffers were tested. 100 mM borate buffer at pH 9.0, the same buffer containing 20% (v/v) ACN or 1% (m/v) SDS to improve the solubility of proteins, 50 mM phosphate buffer at pH 7.0, and an organic buffer (CHES–Tris, 100 mM, pH 9.0) were tested. The highest signals were obtained for 100 mM borate buffer at pH 9.0. These experiments did not allow soybean protein separation. For this reason, the addition of carboxylate-terminated carbosilane dendrimers to the separation buffer was tested to improve protein separation.

The dendrimers selected in this article (see Fig. 1) satisfy the conditions to be good pseudostationary phases due to their stability and solubility under a wide range of analytical conditions and availability at different chemical structures, which may provide different chromatographic selectivity [4]. An initial screening of different generations of dendrimers (G1, G2, and G3) was performed. Then, different concentrations were studied by adding 0.01, 0.05, or 0.1% (m/v) of each dendrimer generation to the 100 mM borate buffer (pH 9.0). The comparison of the results obtained when using the three generations of dendrimers at different concentrations (see Fig. 3) enabled to observe that the number of peaks separated for soybean proteins increased in the presence of dendrimers and also when increasing the percentage of the dendrimer added to the separation buffer. G3 at 0.1% (m/v) was the dendrimer generation which seemed to give the highest peak resolution by observing the electrophoretic profiles obtained. This is in agreement with the fact that the number of carboxylate groups on the dendrimer surface varies according to the dendrimer generation. G3 is the generation which presented the highest number of negative charges (32 on the periphery of the dendrimer), implying the possibility of a major number of interactions of this dendrimer with the positively charged residues of soybean proteins.

The G3 generation was selected as the best nanoadditive for the separation of soybean proteins. For this generation, concentrations of 0.5 and 1% (m/v) were also tested to investigate the effect of the increment of G3 concentration in the separation of soybean proteins. However, the separation of soybean proteins did not improve with dendrimer concentrations higher than 0.1% (m/v). The highest concentration delayed protein peaks, probably due to an increase in the dendrimer–protein interaction with the dendrimer concentration. This effect was previously studied by Shcharbin et al. [18], who found a decrease of protein area with the addition of the G4 PAMAM dendrimer and, with a specific dendrimer/protein molar ratio. These authors interpreted that the peak of the pure protein studied (human serum albumin standard) disappeared due to the full occupation of protein binding centers.

Moreover, due to the high molecular mass of dendrimers, in general, these nanoadditives could increment in excess the time of analysis due to the binding of dendrimers to protein. In this work, analysis times less than 25 min were obtained in all cases. Five peaks were resolved from the two initial peaks in 25 min in the presence of 0.1% G3 carboxylate-terminated carbosilane dendrimers.

3.4. Application of carboxylate-terminated carbosilane dendrimers to the separation of olive seeds proteins

Initially, a method developed for protein extraction from olive stone by our research group [26] was employed. The extraction method consisted on Tris–HCl buffer with 1% (m/v) SDS and 0.5% (m/v) DTT, a centrifugation step to remove the solid remaining, and a protein precipitation step with acetone. However, this extraction procedure was modified in this study because SDS interferers in the dendrimer–protein interactions. This fact was shown using SDS-polyacrylamide gel electrophoresis (PAGE) by Chonco et al. [31] in which dendriplexes formed between water-soluble carbosilane dendrimers and phosphorothioate oligodeoxynucleotides were disrupted by the action of the anionic detergent SDS. For this reason, a separation step proposed by Wang et al. [25] consisting on a chloroform addition after the Tris–HCl buffer extraction was included. This step retained the proteins in the organic phase, removing some SDS interferences and some water soluble compounds. A 90% (v/v) acetone washing step was also included after the protein precipitation with acetone to remove the remaining interferences. Proteins solubilization was other critical step in the extraction procedure due to membrane proteins (olive seeds proteins) do not like to be exposed to aqueous solvents, dissolving easily in the presence of detergents or additives as urea or guanidine. However, as above mentioned, SDS, the universal detergent used for protein solubilization, interferes with the dendrimer–protein interactions established. To demonstrate this fact, the soybean proteins used as standard were dissolved in 0.5 mL of 100 mM Tris–HCl (pH 9.0) with 1% (m/v) SDS. SDS impeded the interaction between the dendrimers

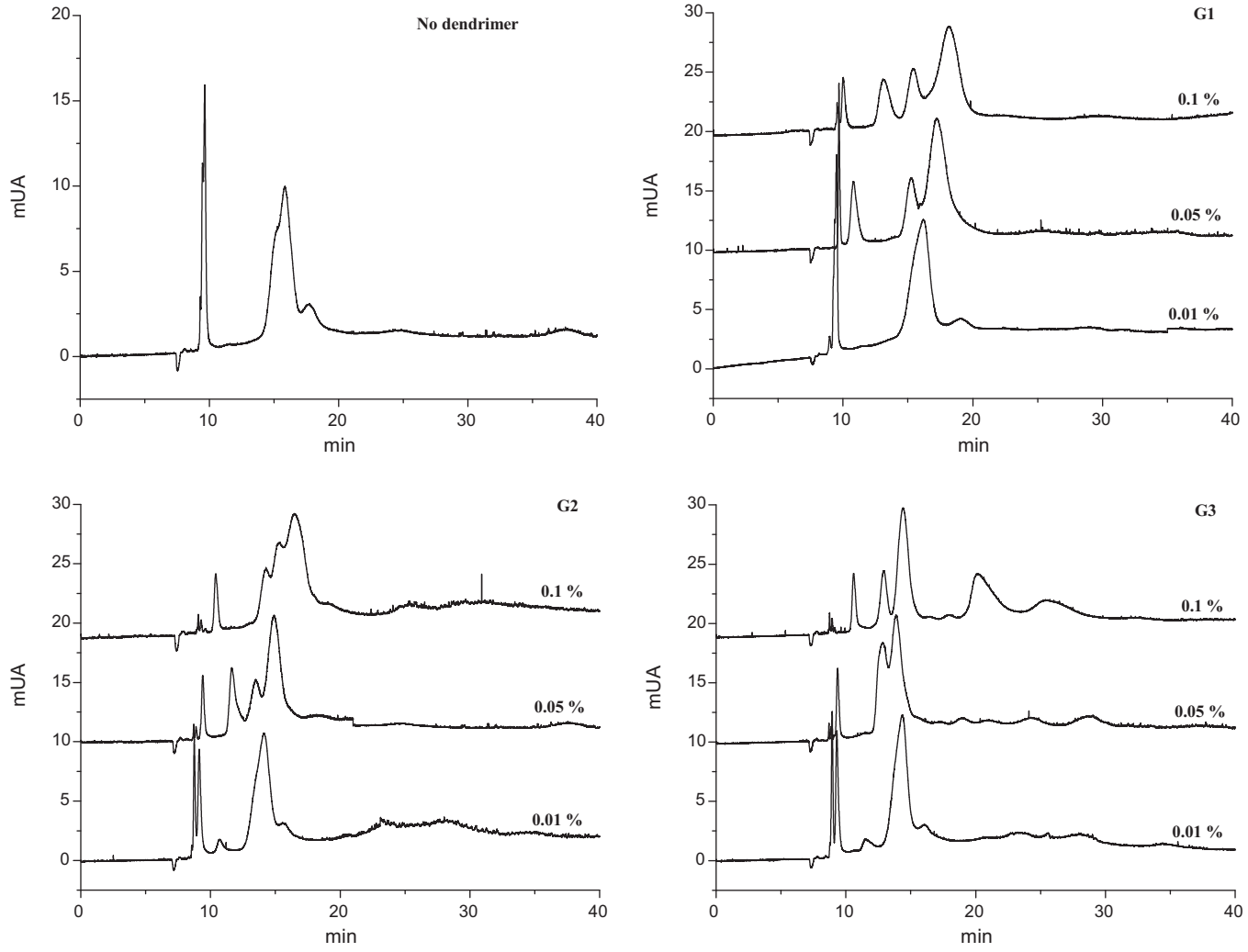

Fig. 3. Comparison of the protein profiles obtained from soybean proteins without and with different carboxylate-terminated carbosilane dendrimer generations (G1, G2, and G3) at three different concentrations (0.01, 0.05, and 0.1% (m/v)). Separation conditions as in Fig. 2. Hydrodynamic injection, 50 mbar for 5 s; UV detection at 254 nm (5 nm bandwidth).

and proteins, not allowing the separation of soybean proteins peaks as when ACN:water (80:20, v/v) was used as solubilization media. For this reason, others additives commonly used for protein solubilization such as 6 M guanidine and 8 M urea (in 10 mM Tris–HCl, pH 8.0) were also tested for olive seeds proteins obtaining similar results as with SDS. Several mixtures of organic solvents with water were also used as solubilization media for the olive seeds extracted proteins: 40% (v/v) ACN, 20% (v/v) methyl ethyl ketone, 50% (v/v) DMSO, 50% (v/v) THF, 50% (v/v) isopropanol, and 100% (v/v) chloroform. None of these media allowed the complete solubilization of olive seeds proteins. For this reason, the same buffer used for the electrophoretic separation was selected and the soluble compounds of the extract obtained from olive seeds were analyzed. Fig. 4 shows the final protein profile obtained for olive seeds proteins using borate buffer (100 mM, pH 9.0) as solubilization medium. In this figure, a comparison of the three generations of dendrimers in the separation buffer is also shown. Based on the previous experience with soybean proteins, the highest concentration (0.1% (m/v)) for each dendrimer generation was tested. It can be observed that the highest generation (G3) at 0.1% (m/v) enabled to improve the protein profile for olive seeds proteins since the highest number of peaks were obtained for this generation dendrimer and with the highest efficiency and resolution. From the initial two peaks obtained with the buffer without dendrimer, six

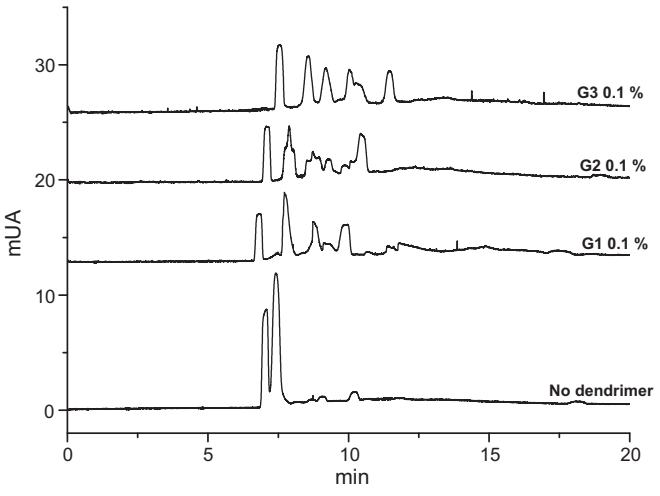

Fig. 4. Comparison of the protein profiles obtained from olive seeds proteins with and without different carboxylate-terminated carbosilane dendrimer generations (G1, G2, and G3) at the same concentration (0.1%, m/v). Separation conditions as in Fig. 3.

peaks (five of them well resolved) were obtained with the separation buffer containing G3 at 0.1% (m/v). The results found here were in agreement with the works available in the literature focused on protein separation by CE using dendrimers in the separation buffer [21,22]. In both articles, improvements in the resolution of protein profiles as a function of dendrimer concentration and generation with a small effect on analysis times were obtained. However, using carboxylate-terminated carbosilane dendrimers, better resolution seems to exist since six peaks were observed from the two initial in comparison with the improvement found in the published works [21,22] consisting in the resolution of co-migrating peaks. These results show that dendrimers can constitute an interesting analytical tool to improve protein profiling for the characterization of vegetable food samples.

4. Concluding remarks

New carboxylate-terminated carbosilane dendrimers have been characterized using CE and potentiometric titration. Moreover, these carboxylate-terminated dendrimers were used as nanoadditives in the separation buffer enabling to improve the separation of different vegetable proteins such as soybean and olive seeds proteins by CE. For olive seeds proteins, from the two initial peaks observed in the protein profiles obtained without dendrimers in the separation buffer, six peaks (five of them well resolved) were obtained with the G3 generation at 0.1% (m/v), without increasing the analysis time. For soybean proteins, five peaks from the initial two peaks were obtained under the same conditions. This fact demonstrated the usefulness of these dendrimers as nanoadditives for the separation of vegetable proteins by EKC. There is currently significant interest in dendrimers as a result of their potential applications, above all, in the field of protein–dendrimer interaction and the increasing possibilities of them. Carboxylate-terminated carbosilane dendrimers used here are proposed as attractive nanoadditives for vegetable protein separations by EKC because they enabled a clear improvement of protein profiles. These results seem to allow its potential application to other proteins. As an example, these anionic carbosilane dendrimers are being studied against HIV showing high antiviral activity as a result of a strong interaction with some viral and/or cell proteins.

Acknowledgements

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