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Synthesis of (±)-3,4-dimethoxybenzyl-4-methyloctanoate as a novel internal standard for capsinoid determination by HPLC-ESI-MS/MS(QTOF)

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Abstract: Capsinoids exhibit health-promoting properties and are therefore compounds of interest for medical and food sciences. They are minor compounds present in relatively high concentrations in only a few number of pepper cultivars. It is desirable to quantify capsinoids to provide selected cultivars with high capsinoid contents, which can then be employed as health food product. Quantifying low concentrations of capsinoids from pepper fruit requires a precise and selective analytical technique such as HPLC coupled to electrospray ionization - mass spectrometry, with development of an internal standard essential. In this work, the synthesis method of a novel compound analogue of capsinoids, the (±)-3,4-dimethoxybenzyl-4-methyloctanoate, which could be a suitable internal standard for capsinoid determination by electrospray ionization - mass spectrometry is described. (±)-3,4-dimethoxybenzyl-4-methyloctanoate was stable under the analysis conditions and exerted chemical and physical properties similar to those of capsinoids. This internal standard will provide an accurate capsinoid determination by electrospray ionization - mass spectrometry, thus facilitating the pepper breeding programs, screening pepper cultivars and a better understanding of capsinoid biosynthetic pathway.

Keywords: Capsiate; dihydrocapsiate, DMBO, fragmentation pattern, HPLC-MS/MS, internal standard, synthesis method

1 Introduction

Capsinoids are analogues to capsaicinoids, these are the main compounds responsible for pungency in pepper fruits (*Capsicum* spp.), and both are exclusively produced by fruits of this genus [1-7]. Although capsinoids also contribute to this effect, they are approximately 1,000 times less pungent than capsaicinoids [7]. Capsinoids are similar in structure to capsaicinoids, with the exception of an ester bond in the place of an amide bond found in capsaicinoids [8]. Until now, three capsinoids have been identified in nature: capsiate, dihydrocapsiate, and nordihydrocapsiate, with the first two the most common [8,9]. Both, capsaicinoids and capsinoids are powerful biocompounds with health-promoting properties, such as anti-oxidant, anti-inflammatory, anti-obesity, and analgesic activities [10-13]. They are also used in arthritis treatments, digestive disorders or in some diseases like cancer, among others [14-17]. The advantage of capsinoids over capsaicinoids is that these do not present side effects like irritancy or a burning feeling [18,19]. Therefore, capsinoids could have a greater application range both in medical field and in food sciences, in the development of new drugs for long-term treatments or functional foods. In this regard, identifying pepper cultivars with high capsinoid content becomes

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really attractive for human consumption, pharmaceutical purposes and pepper breeding programs.

The analytical techniques used, routinely in laboratories, for capsinoid determination are high performance liquid chromatography (HPLC) coupled to UV or PDA/DAD detectors [2-4,20]. Since capsinoids and capsaicinoids are measured at the same wavelength (280 nm), their concentration could be overestimated by these techniques, which are based only on retention time and UV spectra, and could assign erroneously a peak to a given capsinoid. A promising high-resolution detection technique such as electrospray ionization (ESI)-mass spectrometry (MS) in combination with HPLC, would enable a more accurate, precise, and sensitive analysis of these compounds. This technique allows an unequivocal identification of compounds by exact mass-to-charge ratio (*m*/*z*), molecular formula and fragmentation pattern, all of them distinctive features for each compound [21-24]. Nevertheless, the addition of an internal standard (IS) to the sample is of crucial importance in order to improve the analytical parameters, avoid ionization fluctuations of analytes, and correct errors in detection [21-27]. Currently, there are not any commercially available internal standards for capsinoid analysis by HPLC-ESI-MS. Isotope-labeled analogues could be the internal standards. However, they have disadvantages, such as their scarce availability and high price. Thus, chemical synthesis is an interesting alternative to isotopic labelling for obtaining new synthetic analogues to capsinoids [22, 28].

In this report, we describe for first time a simple synthesis approach of the (±)-3,4-dimethoxybenzyl-4-methyloctanoate ((±)-DMBO), a novel synthetic analogue to capsinoids, and their potential use as IS for capsiate and dihydrocapsiate determination by HPLC-ESI-MS(QTOF).

2 Materials and methods

2.1 Chemicals and reagents

For (±)-DMBO synthesis: 3,4-dimethoxybenzaldehyde (99%) (**1**), and (±)-4-methyloctanoic acid (98%) (**3**), and di-isobutyl aluminium hydride (1 mol L^{-1} in toluene) (DIBAL) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium sulphate anhydrous (99%), dehydrated pyridine (99%), and tetrahydrofuran (THF) (99.5%) were purchased from Panreac Química S.L.U. (Barcelona, Spain). Ethyl acetate, methanol, and hexane, all of them HPLC grade, were procured from Scharlab S.L. (Barcelona, Spain). Thionyl chloride ($SOCl_2$) (99%) was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). Capsiate and dihydrocapsiate standards were supplied by the Department of Organic Chemistry at the University of Cádiz, Spain. All standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA).

2.2 General experimental procedures

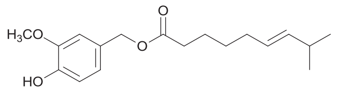
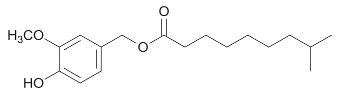
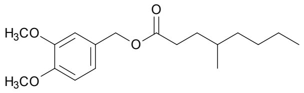
The synthesis of (±)-DMBO (**5**) was accomplished in three stages: reduction of the 3,4-dimethoxybenzaldehyde (**1**) carbonyl group, chloride formation from (±)-4-methyloctanoic acid (**3**) and esterification of 3,4-dimethoxybenzyl alcohol (**2**) with (±)-4-methyloctanoyl chloride (**4**).

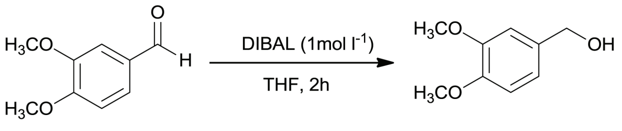
The (±)-DMBO purity was determined by ^1H NMR and ^{13}C NMR analysis. ^1H and ^{13}C spectra were recorded using deuterated chloroform ($CDCl_3$) (99.8%, Sigma-Aldrich Chemical Co. St. Louis, MO, USA) as the solvent, on an Agilent INOVA spectrometer (Agilent Technologies, Santa Clara, CA, USA) at 499.719 and 125.654 MHz, respectively. The resonances of residual chloroform for ^1H and ^{13}C were set to δ_H 7.25 ppm and δ_C 77.00 ppm, respectively, and used as internal reference. UV-Vis spectra were obtained by using a Varian Cary 50 BIO spectrophotometer (Agilent Technologies) with chloroform as the solvent. General IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 2 spectrophotometer (PerkinElmer, Waltham, MA, USA). The reactions were monitored by thin-layer chromatography (TLC) on silica gel (F245 Merck plates).

2.3 HPLC-ESI-MS and ESI-MS/MS(QTOF) analysis

Individual standard solutions in 60% methanol (20 µmol L^{-1} for (±)-DMBO and 10 µmol L^{-1} for capsiate and dihydrocapsiate) were introduced by direct injection with a syringe pump (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) operating at 4 µL min^{-1}, in a Quadrupole Time-of-Flight (QTOF) mass spectrometer equipped with an Electrospray Ionization Source (ESI) (MicrOTOF-Q, Bruker Daltonics, Bremen, Germany). ESI-MS(QTOF) and ESI-MS/MS(QTOF) analysis were carried out in positive-ion mode, with capillary and endplate offset voltages of 4500 and -500 V, respectively, and using N_2 as the collision gas. The collision cell energy was set to 10 eV, with an isolation width for the precursor ion of 2 mass-to-charge ratio (*m*/*z*)

Table 1: Common name, chemical formula, molecular weight, and [M+Na]$^+$ of capsiate, (±)-DMBO, and dihydrocapsiate.

Common Name	Chemical Structure	Chemical Formula	Molecular Weight	Measured [M+Na]$^+$
Capsiate		$C_{18}H_{26}O_4$	306.3966	329.1678
Dihydrocapsiate		$C_{18}H_{28}O_4$	308.4125	331.1879
(±)-DMBO		$C_{18}H_{28}O_4$	308.4125	331.1855



(1) (2)

Figure 1: Step 1 of the synthesis procedure of (±)-DMBO. Reduction of 3,4-dimethoxybenzaldehyde (**1**) to 3,4-dimethoxybenzyl alcohol (**2**).

units. The nebulizer gas (N_2) pressure, the drying gas (N_2) flow rate and the drying gas temperature were 1.6 bar, 8.0 L min^{-1} and 200 ºC, respectively. Spectra were acquired in the *m/z* 50–800 range. The mass axis was calibrated by using Na-formate adducts [10 mmol L^{-1} NaOH, 2.5% (v/v) formic acid and 50% (v/v) 2-propanol] that were introduced through a divert valve at the beginning of each direct injection. For MS/MS spectra the [M+Na]$^+$ ions with *m/z* values of 329 and 331 *m/z* for capsiate and dihydrocapsiate and (±)-DMBO, respectively, were chosen as precursors, with an isolation width of 4 *m/z* units and an amplitude voltage of 0.45 V. The structures of capsiate, (±)-DMBO, and dihydrocapsiate are shown in Table 1. Bruker Daltonik software packages micrOTOF Control v.2.3 and HyStar v.3.2 were used to control the system. Data Analysis v.4.0 was used to process data. Capsiate, (±)-DMBO, and dihydrocapsiate standards were detected by coupling the HPLC instrument to the QTOF mass spectrometer. Chromatographic separation was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbron, Germany) equipped with an isocratic pump, an autosampler, and a thermostated column compartment. The column used was an analytical HPLC C_{18} column (Symmetry® C_{18}, 2.1 x 150 mm; 3.5 µm spherical particle size, Waters, Milford, MA, USA) protected by a guard column (Symmetry® C_{18}, 2.1 mm i.d. x 10 mm length, 3.5 µm spherical particle size, Waters). The HPLC conditions were methanol (solvent A) and Milli-Q water acidified with 0.1% (v/v) acetic acid (solvent B) as eluents, 0.2 µL min^{-1} as flow rate, 25 ºC as column temperature, and 5 µL as injection volume. The elution program was set according to the following gradient: 0-7 min, 60% B; 7-15 min, 60-70% B; 15-52 min, 70-100% B.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and discussion

3.1 (±)-DMBO Synthesis procedure (5)

3.1.1 Reduction of the 3,4-dimethoxybenzaldehyde (1) carbonyl group

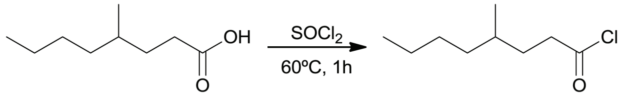
The initial reaction stage involved the reduction of the carbonyl group of 3,4-dimethoxybenzaldehyde (**1**) to its corresponding alcohol (**2**) (Figure 1). The reduction of the carbonyl group was performed by using DIBAL (1 mol L^{-1} in toluene). The compound (**1**) (3.0042 g, 0.0181 mol) was dissolved in 40 mL of dry THF. Subsequently, 2 equivalents of DIBAL (1 mol L^{-1} in toluene) were added slowly, with a syringe, in an ice bath, and the reaction mixture was stirred, under argon, at ambient temperature, for 2 h. The reaction was monitored by TLC (eluent: 20% ethyl acetate, 80% hexane; chromogenic reagent: anisaldehyde). Then, the reaction was stopped with water and after purification of the reaction mixture, by column chromatography (eluent: 20% ethyl acetate, 80% hexane; chromogenic reagent: anisaldehyde), and (**2**) was obtained with a yield of 77.16%.

3.1.2 Chloride formation from (±)-4-methyloctanoic acid (3)

The (±)-4-methyloctanoic acid (**3**) was transformed into (±)-4-methyloctanoyl chloride (**4**), in order to subsequently perform the acylation of this compound (**2**) (Figure 2). To do so, 2 equivalents of (**3**) (4.4307 g, 0.028 mol) were placed in a 50-mL round-bottom flask into which argon was introduced. An amount of $SOCl_2$, sufficient to dissolve the acid, was added very slowly, dropwise from a syringe, and stirred magnetically. After the addition of $SOCl_2$, the reaction mixture was heated at 60 ºC for 1 h. Subsequently, the excess of $SOCl_2$ excess was eliminated in vacuo, and the transparent oil of (**4**) was obtained. This compound (**4**) was dissolved in dry THF for the subsequent acylation with compound (**2**).

3.1.3 Esterification of 3,4-dimethoxybenzyl alcohol (2) with (±)-4-methyloctanoyl chloride (4)

The (±)-3,4-dimethoxybenzyl-4-methyloctanoate ((±)-DMBO) (**5**) was prepared by the acylation of the 3,4-dimethoxybenzyl alcohol ring (**2**) using a (±)-4-methyloctanoyl chloride chain (**4**) (Figure 3). The final product (±)-DMBO (**5**) is a racemic mixture since the synthesis has been started from the racemic product (±)-4-methylocatanoic acid (**3**) and the proposed synthesis is not stereoselective. Compound (**2**) (2.3321 g, 0.0140 mol) was dissolved in 20-25 mL of dry pyridine in a 50-mL round-bottom flask under a protective atmosphere with argon. Then, 2 equivalents of (**4**) were added slowly to this solution and agitated for 18 h. The reaction was monitored by TLC (eluent: 20% ethyl acetate, 80% hexane; chromogenic reagent: anisaldehyde) up to the reaction´s completion.



(3) (4)

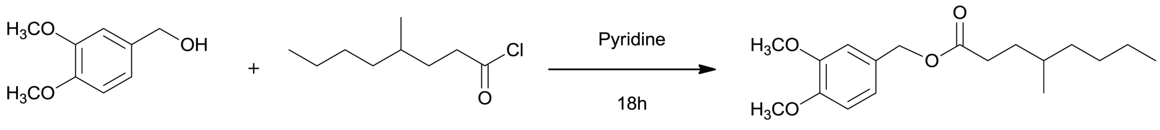
Figure 2: Step 2 of the synthesis procedure of (±)-DMBO. Preparation of (±)-4-methyloctanoyl chloride (**4**) from (±)-4-methyloctanoic acid (**3**).

The (±)-DMBO esterification step was simpler than that reported for capsinoid and capsaicinoids standards synthesis [29]. Through this synthesis procedure, (±)-DMBO (**5**) was obtained, after purification of the reaction mixture, with a yield of 95% and a purity ≥ 98%. The overall yield obtained for the synthesis of (±)-DBMO starting from the initial material has therefore been 73.15%. This yield was higher than those obtained by other synthesis procedures previously reported for capsinoid and capsaicinoid standards [12,29-33]. Thus, this procedure would be an efficient alternative to other synthesis methodologies for capsinoid derivatives, such as enzymatic catalysis or labeled compound synthesis, due to its lower cost and higher yield.

3.2 Chemical characterization of (±)-DMBO (5)

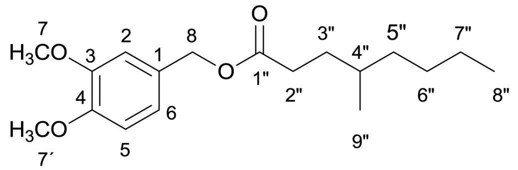
The formation of (±)-DMBO was confirmed by ^{1}H NMR and ^{13}C NMR analysis. Since (±)-DMBO (**5**) (Figure 4) has never been previously described in the bibliography, its chemical characterization is presented below:

Yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm) (Assignments with identical superscripts are interchangeable) (S1 _Figure): 6.91 (dd, 1H, J = 2.0 Hz, J = 8.1 Hz, 6), 6.87 (bd, 1H, J = 1.9 Hz, 2), 6.83 (bd, 1H, J = 8.1 Hz, 5), 5.03 (s, 2H, 8), 3.87 (s^a, 3H, 7), 3.86 (s^a, 3H, 7´), 2.38-2.26 (m, 2H, 2”), 1.69-1.63 (m, 1H, 3a”), 1.47-1.41 (m, 1H, 3b”), 1.41-1.35 (m, 1H, 4”), 1.30-1.17 (m, 1H, 5a”), 1.30-1.17 (m, 2H, 7”), 1.30-1.17 (m, 2H, 6”), 1.12-1.07 (m, 1H, 5b”), 0.86 (t, 3H, J = 6.4 Hz, 8”), 0.84 (d, 3H, J = 6.5 Hz, 9”); ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 173.98 (1”), 148.99^b (4), 148.91^b (3), 128.59 (1), 121.17 (6), 111.70 (2), 110.94 (5), 66.14 (8), 55.85^c (7), 55.81^c (7´), 36.27 (5”), 32.31 (4”), 32.12 (2”), 31.85 (3”), 29.09 (6”), 22.87 (7”), 19.24 (9”), 14.05 (8”); IR (cm^{-1}): 2956, 1735,



(2) (4) (5)

Figure 3: Step 3 of the synthesis procedure of (±)-DMBO. Procedure for the preparation of (±)-DMBO (**5**) from the acylation of 3,4-dimethoxybenzyl alcohol (**2**) with (±)-4-methyloctanoyl chloride (**4**).



(5)

Figure 4: Chemical structure of (±)-DMBO (5). Chemical characterization of (±)-DMBO by ^{1}H NMR and ^{13}C NMR analysis.

1519, 1162; λ_{max} (nm) 279.9; ε (L/mol·cm^{-1}) 338; HRMS (ESI) calculated for $C_{18}H_{28}O_4$ $[M+Na]^+$: 331.1879, found: 331.1884.

3.3 HPLC-ESI-MS(QTOF) and HPLC-ESI-MS/MS(QTOF) analysis of capsiate, (±)-DMBO, and dihydrocapsiate standards

In order to evaluate the suitability of (±)-DMBO as an IS for capsinoid determination by ESI-mass spectrometry, its retention time and ionization behaviour were determined by HPLC-ESI-MS(QTOF) and HPLC-ESI-MS/MS(QTOF) and compared to those of capsiate and dihydrocapsiate standards.

The MS spectra of capsiate, (±)-DMBO, and dihydrocapsiate were acquired, in positive ion mode, by direct injection of the standard solutions on the QTOF mass analyser. The major peaks observed in the MS spectra corresponding with the $[M+Na]^+$ ions were at the *m/z* 329.1678 for capsiate, 331.1879 for dihydrocapsiate and 331.1855 for (±)-DMBO (Table 1). These molecular ions were in agreement with the calculated *m/z* 329.1723 for capsiate and 331.1880 for (±)-DMBO and dihydrocapsiate; therefore, they were selected as precursors for ESI-MS/MS experiments. Figure 5 shows the MS/MS spectra obtained for capsiate, (±)-DMBO, and dihydrocapsiate. For both capsinoid standards, the most intense peak was observed at *m/z* 159.0375 for capsiate and 159.0418 for dihydrocapsiate, in accordance with the calculated *m/z* 159.0417, corresponding to sodiated vanillyl ring. Other minor product ions detected at *m/z* 137.0570 and 137.0605, in conformity with the calculated *m/z* 137.0597, corresponding to the protonated vanillyl ring of capsiate and dihydrocapsiate, respectively, and generated by the cleavage of the C7-O8 bond of these molecules (Figure 5, A and B). On (±)-DMBO MS/MS spectrum, the most intense peak was observed at *m/z* 151.0750 (Figure 5C), in agreement with the calculated *m/z* 151.0754 for the expected vanillyl-type ring resulting from fragmentation at the ester bond. The ionization behaviour and break site of (±)-DMBO molecules were similar to those

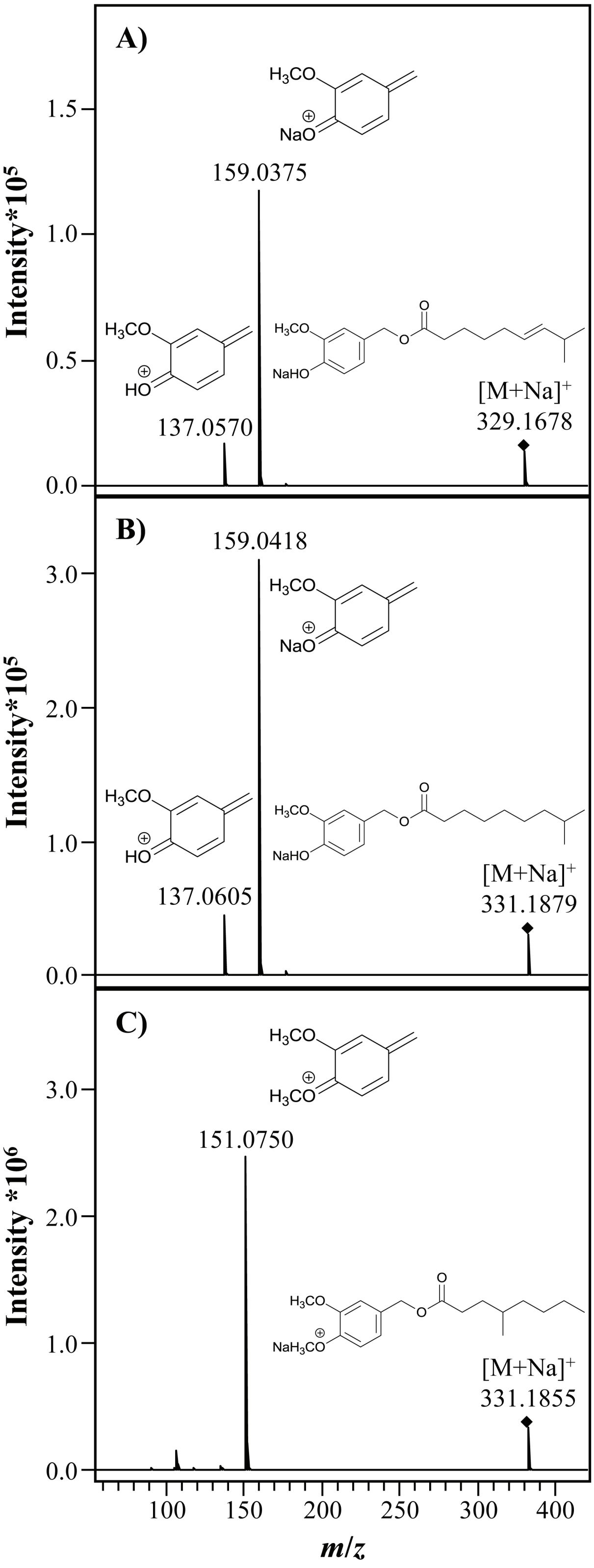


Figure 5: ESI-MS/MS spectra in positive ion mode of capsiate, dihydrocapsiate and (±)-DMBO. The individual standard solutions in 60% methanol of 10 µmol L^{-1} capsiate (A) and dihydrocapsiate (B), and 20 µmol L^{-1} (±)-DMBO (C) were acquired by direct injection on the QTOF mass analyser.

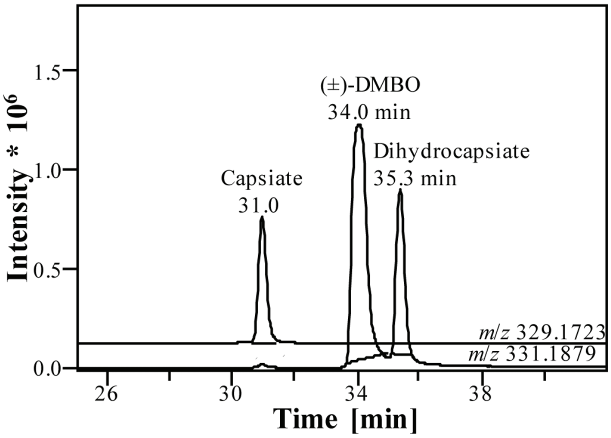


Figure 6: HPLC-ESI-MS(QTOF) chromatograms of capsiate, (±)-DMBO and dihydrocapsiate standards. Ion chromatograms were extracted for the exact *m/z* values corresponding to the $[M+Na]^+$ species of capsiate, (±)-DMBO and dihydrocapsiate.

shared by capsinoid standards. MS/MS spectra obtained for (±)-DMBO, capsiate, and dihydrocapsiate matched well with the typical fragmentation pattern for capsinoids [34] and capsaicinoids [35].

Ion chromatograms were extracted for the exact *m/z* values corresponding to the $[M+Na]^+$ species of capsiate, (±)-DMBO, and dihydrocapsiate (Figure 6). The peaks of the capsiate, (±)-DMBO, and dihydrocapsiate standards showed their elution times at 31.0, 34.0, and 35.3 min, respectively. Since the chromatogram traces corresponding to *m/z* 331 were extracted for (±)-DMBO and dihydrocapsiate, the resolution (Rs) of these two compounds was calculated in order to confirm the correct separation of both compounds. The Rs of dihydrocapsiate and (±)-DMBO (defined as 2 times the separation between peaks divided by the sum of the peak base widths) was 1.2, a value that can be considered as adequate.

The potentiality of using (±)-DMBO as IS in capsinoid determination by ESI-mass spectrometry was supported by the following facts i) it is a capsinoid analogue not occurring naturally in *Capsicum* species, ii) the ionization behaviour of (±)-DMBO in ESI mass is similar to that of capsiate and dihydrocapsiate, and iii) its retention time is similar to that of capsiate and dihydrocapsiate, (±)-DMBO eluting between the two analytes but being able to be separated from dihydrocapsiate.

4 Conclusion

In this study, a (±)-DMBO synthesis procedure has been described. The synthesis procedure developed is simple, economical, and achievable in any basic chemistry laboratory, and it also allows the obtaining of yields greater than 95%.

(±)-DMBO could be a potential IS for capsinoid determination because: (i) it is not present in pepper fruits; (ii) it is stable under analysis conditions; (iii) it elutes between capsiate and dihydrocapsiate; (iv) its chemical structure is closely related to that of capsinoids; (v) and its ionization behaviour and fragmentation pattern is shared by capsiate and dihydrocapsiate.

As far as we know, there is not any other IS available for capsinoid determination by HPLC-ESI-MS/MS(QTOF) analysis. Synthetic analogues have already been successfully used as IS for another compound determination by ESI-MS techniques [36-37]. We have described for first time a simple synthesis approach of a capsinoid analogue suitable for IS. The development of a new IS will allow an accurate quantification of capsinoid by HPLC-ESI-MS, a useful tool for screening and selecting of cultivars containing capsinoids in pepper breeding programs and for gaining understanding of the metabolism of capsinoids.

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Conflict of interest: Authors state no conflict of interest.

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