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Short communication

Validated high-performance liquid chromatographic method for quantitation of neohesperidine dihydrochalcone in foodstuffs

H. Montijano[a], F. Borrego[a,*], I. Canales[a], F.A. Tomás-Barberán[b]

[a]*Zoster, S.A. (Grupo Ferrer), Raiguero 143, 30588, Zeneta (Murcia), Spain*

[b]*CEBAS (CSIC), PO Box 4195, 30800, Murcia, Spain*

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Abstract

An analytical method to detect and quantitate neohesperidine dihydrochalcone in foodstuffs has been developed and validated in soft-drink applications. The method was shown to be sufficiently precise, accurate, selective and rugged in quantitating neohesperidine DC both at flavouring (1–5 mg/kg) and sweetening (5–50 mg/kg) levels. Applications of the method to determine neohesperidine DC in foodstuffs other than soft-drinks is also described.

Keywords: Neohesperidine dihydrochalcone

1. Introduction

Neohesperidine dihydrochalcone (hereinafter referred as neohesperidine DC) is an intense sweetener, 1800 times sweeter than sucrose at threshold concentrations and about 400 times sweeter when compared to a 6% sucrose solution [1]. It also contributes flavour enhancing and modifying properties, which are apparent even at neohesperidine DC levels at which there is no significant contribution to sweetness (1–5 mg/kg) [2–4].

Neohesperidine DC has been recently authorized in the European Union as an intense sweetener in a wide range of energy-reduced or non-added-sugar foodstuffs [5], and also as a flavour enhancer in an additional number of foods at concentrations below the sweetness threshold [6]. Therefore, it is important that neohesperidine DC can be detected in products at the proposed levels of use, and that it be possible to analytically differentiate between flavouring use-levels and sweetening use-levels.

HPLC methods to quantitate neohesperidine DC in foodstuffs have been previously reported in the literature [7–9] and recently reviewed [3]. These studies, while useful starting points, were limited to one single product (grapefruit juice) [7], or studied some foods and beverages containing unrealistically high neohesperidine DC concentrations in others [9].

A fully validated HPLC method to detect and quantitate neohesperidine DC both at sweetening and flavouring levels is still lacking. This paper reports such a method.

2. Experimental

2.1. Reagents

Neohesperidine DC was from Zoster S.A. (Murcia, Spain). HPLC grade acetonitrile, glacial acetic

*Corresponding author.

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acid, reagent grade dimethyl sulphoxide (DMSO) and methanol were from Merck (Darmstadt, Germany).

2.2. Food samples processing

Standard validation parameters have been characterized in a carbonated orange beverage. The method has also been applied to analysis of neohesperidine DC in skimmed plain yogurt (containing 2 and 10 mg/kg neohesperidine DC), lemon-flavoured hard candies (5 and 50 mg/kg) and low-fat minarine (3 mg/kg).

Control samples without neohesperidine DC were processed and analyzed in all cases under the same conditions as for the test samples.

The processing of the carbonated soft drink consisted in sonicating it in a glass beaker for 30 min in order to remove all carbon dioxide. A 5-g amount of yogurt was extracted with four portions (5 ml each) of a mixture of DMSO and methanol (1:1, v/v) and the mixture stirred and centrifuged (Centromix, Selecta) for 10 min at 6000 rpm. All the liquids were combined and made up to a volume of 25 ml. A 25-g portion of minarine was melted by heating at 40°C and extracted with 25 ml DMSO. The bottom DMSO layer was allowed to separate in a decantation funnel, collected and the final volume measured. A 2-g portion of crushed lemon sweet was extracted with two portions (5 and 2 ml respectively) of a methanol–water mixture (1:1, v/v). Both fractions were combined and made up to a volume of 10 ml and the total mixture stirred for 10 min.

Prior to HPLC analysis, all extracts were centrifuged at 6000 rpm for 10 min.

2.3. HPLC analysis

Neohesperidine DC was determined in each food and beverage sample by HPLC in a Merck-Hitachi liquid chromatograph, equipped with a L-6200 pump, a L-4000 UV detector, a D 2500 integrator and an AS-2000 A autosampler, under the following conditions: Lichrospher RP C_{18} column (5 μm particle size, 4×150 mm I.D.); solvent flow-rate, 1 ml/min; column temperature, 30°C; UV detection at 282 nm; and mobile phase acetonitrile–acidified water (containing 0.5% acetic acid, 20:80, v/v). Injection volume was 10 μl. With this procedure neohesperidine DC eluted at about 9 min.

2.4. Quantitation of neohesperidine DC

Neohesperidine DC content in all samples was determined by the external standard method. Detector response to neohesperidine DC in different media was checked by assaying three replicate samples of neohesperidine DC (10 mg/l) in each of the following solvents: water, DMSO, methanol, DMSO–water (1:1, v/v), methanol–water (1:1, v/v) and DMSO–methanol (1:1, v/v).

2.5. Validation procedure

System precision measurements were made from ten replicate injections of a single orange soft-drink sample preparation, containing 10 mg/l neohesperidine DC. Method precision was measured by analysing six replicate soft-drink samples containing neohesperidine DC at 4 and 10 mg/l.

Ruggedness was measured with 6 replicate injections of one sample containing 4 mg/l and 10 mg/l neohesperidine DC. Analyses were performed on four different days with different standard solutions, mobile phases and by two different analysts and in two equipments.

To check method accuracy, soft-drink samples were prepared containing 0.5, 1, 2, 3, 4, 8, 10 and 12 mg/l neohesperidine DC (to cover both the flavouring and sweetening range) in triplicate and analyzed three times.

Method selectivity was shown by stressing a sample of carbonated orange soft-drink containing 10 mg/l neohesperidine DC by means of heating at 90°C during 24 h at pH 2.0. Control samples without neohesperidine DC were evaluated to ensure there was no interference in the analysis.

3. Results and discussion

3.1. Preliminary work

Initial HPLC working conditions (columns, mobile phase) were selected on the basis of previously published works (see Ref. [3] for a recent review),

our own experience in quality control and stability studies performed in food systems [10,11]. In a preliminary experimental phase, these initial conditions were subjected to slight changes to see the impact, if any, on neohesperidine DC quantitation. Increase of column temperature from 30 to 40°C and change in mobile phase composition from 20 to 25% acetonitrile had no significant impact in neohesperidine DC quantitation (data not shown), although this remarkably influenced retention time.

3.2. Validation procedure in soft-drinks

Validation requires proof of accuracy, system and method precision, selectivity under stressed conditions along with some indications of ruggedness (inter-lab, inter-analyst or inter-day adequacy of performance).

The results showed that system and method precision, as well as ruggedness, were within the acceptable (R.S.D.<2%) range, also indicating slightly better method reproducibility at sweetenening (R.S.D.=0.81%) than at flavouring (R.S.D.=1.94%) concentrations, and suggesting that the method would be easiliy transferable between different laboratories.

Method accuracy results, expressed as percent recoveries, are summarized in Table 1. Very acceptable mean recoveries and R.S.D. values are shown, although this latter parameter showed values slightly higher than desirable at the lowest tested concentrations (0.5–3 mg/l). This fact has however little, if any, practical relevance because even these low concentrations can be quantified with acceptable accuracy. All tested concentrations are statistically different with a value of $p=0.01$.

Neohesperidine DC is a glycosidic flavonoid which can hydrolyze under extreme combined conditions of high acidity and temperature to yield a blend of hesperetin dihydrochalcone, hesperetin dihydrochalcone-4′-β-D-glucoside and the sugars rhamnose and glucose [1]. The analysis of a soft-drink stressed sample under the conditions stated in the Section 2 showed retention times of 9, 10.5 and 34 min for neohesperidine DC, hesperetin dihydrochalcone-4′-β-D-glucoside and hesperetin dihydrochalcone respectively. Selectivity in the method was demonstrated by lack of interference from degradation products in acid and heat stressed samples.

In addition, no analytical interferences have been detected during analysis of more than 20 different types of flavoured- and juice-based soft-drinks (containing 10 mg/l neohesperidine DC) at our laboratory (data not shown).

3.3. Quantitation of neohesperidine DC in foodstuffs other than soft-drinks

3.3.1. Chromatographical response of neohesperidine DC in different solvents

The results obtained are summarized in Table 2 and are expressed as percentages relative to response in DMSO. As can be seen, differences in response between pure solvents and the binary blends were very small and of no practical relevance. However, response in water was about 30% lower than in any other solvent, thus illustrating the importance of keeping constant solvent composition in the food extract and standard solution.

Table 1
Accuracy results for neohesperidine DC analysis in an orange soft-drink at flavouring and sweeting levels

Added (mg/l)	Mean recovery (%)	R.S.D. (%)
0.5	107.2	3.8
1.0	103.6	3.0
2.0	101.6	3.1
3.0	100.9	2.5
4.1	97.4	0.8
8.3	101.5	0.9
10.4	102.0	0.9
12.5	102.3	0.4

Table 2
UV detector response to neohesperidine DC dissolved in different media (±95% confidence limits)

Solvent	Detector response (%)
DMSO	100.0±0.4
Methanol	99.4±4.0
DMSO–water (1:1, v/v)	98.3±1.2
DMSO–methanol (1:1, v/v)	97.8±0.9
Methanol–water (1:1, v/v)	96.3±0.9
Water	71.8±2.8

3.3.2. Application to foods other than beverages

Non-alcoholic beverages is the major application field for intense sweeteners. For this reason, the validation work has been performed in a carbonated orange soft-drink. The method has been applied to other foods, in which the use of neohesperidine DC has also been authorized.

The following foodstuffs have been selected as representative of neohesperidine DC applications: plain yogurt, lemon hard candy and minarine. In the former two products analyses of neohesperidine DC have been evaluated at both flavouring and sweetening levels, while in margarine only the analysis at flavouring concentration has been checked accordingly to its regulatory status [6].

Having in mind the differences in detector response detected in various solvents, standard neohesperidine DC solution was prepared using the same solvent composition and at the concentration expected in each beverage or food sample extract.

Table 3 summarizes the results obtained in all samples tested. Neohesperidine DC concentrations represent mean values from replicate analysis (two samples at each neohesperidine DC concentration and three injections per sample). Acceptable recovery values (>90%) have been obtained in all tested food samples both at flavouring and sweetening levels. Even in the case of complex food samples containing a very low concentration of neohesperidine DC (minarine), acceptable recovery has been found with a single liquid–liquid extraction step.

Table 3
Results for neohesperidine DC quantitation in foodstuffs other than carbonated beverages (±95% confidence limits)

Food sample	Added (mg/kg)	Mean found (mg/kg)
Plain yogurt	2.3	2.4±0.3
	10.3	10.1±0.2
Lemon hard candy	6.0	5.9±0.5
	58.3	58.8±0.6
Minarine	2.9	2.6±0.4

4. Conclusions

The method developed makes the accurate, precise and selective determination of neohesperidine DC in soft-drinks and other foodstuffs possible. Unlike previous works, this method has been tested in a wide range of foodstuffs and with conditions of use in mind, i.e. as a sweetener and flavour modifier, which are representative of the recent advances in the regulatory status of this substance.

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