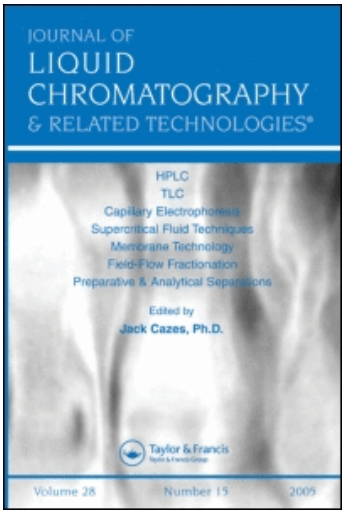

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ANALYSIS OF COMMERCIAL NEOHESPERIDIN DIHYDROCHALCONE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC method for checking the purity of commercial neohesperidin dihydrochalcone has been developed and validated. The optimised method was conducted by using an RP C_{18} stationary phase, 20% acetonitrile acidified with acetic acid as mobile phase, 1 mL/min flow rate, spectrophotometric detection at 282 nm, and a temperature of 25°C. Under these conditions, neohesperidin DC showed a retention time of 17.4 min. Different structurally related flavonoids were added to the sample for selectivity determination. Linearity of the method was proved in the range 0-500 mg/L. Sample preparation was optimised and relevant analytical parameters (accuracy, precision, repeatability, reproducibility) determined.

INTRODUCTION

Neohesperindin dihydrochalcone (neohesperidin DC) is an intense sweetener which is about 1800 times sweeter than sucrose at threshold levels and about 400 times sweeter in comparison to a 6% sucrose solution.[1]

Its sweetening profile in water is characteristical by a delay before its maximum intensity is reached, followed by a menthol- or licorice-like lingering aftertaste.[2] When used at low concentrations in combination with other intense or bulk sweeteners, neohesperidin DC enhances the quality of the sweetness given to the food, contributing beneficially to its flavour and mouthfeel and providing synergistic effects.[3,4]

International acceptance of neohesperidin DC was manifested by a favourable assessment and the allocation of an ADI by the Scientific Committee for Food of the European Union. In fact, it has been recently authorised in the EU as an intense sweetener[5] and flavour enhancer.[6]

Although official purity criteria have also been established,[7] no mention is made of the analytical method proposed to control the purity of the sweetener. Therefore, the development of a validated method to ensure that commercial products meet official specifications is of industrial and scientific interest.

The objective of the present work has been the development and validation of such a procedure using HPLC, for use in the analysis of the commercial product's purity.

MATERIALS AND METHODS

Materials

Neohesperidin DC (NHDC) and related compounds were supplied by Zoster S.A. (Raiguero, 143, Zeneta, E-30588, Murcia, Spain). The related compounds were: phloroacetophenone neohesperidoside (FANH), naringin (NA), neohesperidin (NH), naringin dihydrochalcone (NADC), hesperidin dihydrochalcone (HEDC), hesperetin dihydrochalcone glucoside (HPDCG), and hesperetin dihydrochalcone (HPDC). Acetonitrile (ACN) and methanol were from Merck and dimethyl sulphoxide (DMSO) was from Romil. All solvents were HPLC grade. Acetic and phosphoric acids employed for mobile phase acidification were from Fluka and Merck, respectively. Water was double distilled and purified through a Millipore system (Milli-Q).

Equipment

HPLC analysis was performed on a Shimadzu HPLC system equipped with two LC-6A pumps, a UV-Vis SPD-M6A diode array detector and a SIL 9A automatic injector. For reproducibility determination two HPLC systems from different laboratories were used: i) a Hitachi HPLC system equipped with a L6200A pump, UV-Vis L 4250 detector, AS 2000A automatic injector and D 2500 integrator; and ii) a Shimadzu HPLC equipped with two LC8A pumps, UV-vis SPD-6AV detector and C-R4A integrator. The columns employed with this equipment were Lichrospher 100 RP-C_{18} 5μm (120 x 4 mm) columns from Merck. Assay temperature was 25°C.

Sample Preparation

A standard sample of neohesperidin DC (200 mg/L) was prepared by dissolving 50 mg of commercial neohesperidin DC (L29024) in 250 mL of solvent (DMSO, 50% methanol or 50% ACN, depending on the assay). The other related compounds were added at a lower concentration (4 mg/L) to simulate the presence of impurities. 4 mg/L was sufficient for quantitation and identification of each added compund. Four different samples were analysed: neohesperidin DC dissolved in DMSO (sample P); and neohesperidin DC plus all related compounds dissolved in DMSO (sample A), in 50% methanol (sample B), and in 50% ACN (sample C). For calibration, precision, and accuracy determinations, neohesperidin DC dissolved in DMSO was analysed at different concentrations. For selectivity determinations, a standard of neohesperidin DC purified by preparative HPLC was employed as reference.

Method Development

The mobile phases assayed were binary mixtures of methanol:water and ACN:water, acidified with acetic acid (50 mM). Samples B and C were assayed only with methanol:water and ACN:water, respectively, while samples prepared in DMSO (P and A) were assayed with all the elution systems. All analyses were performed in triplicate. Chromatographic parameters were calculated according to.[8,9] Optimum mobile phase acidification was selected from the analysis of neohesperidin DC in 20% ACN acidified with both acetic (50 mM) and phosphoric (50 mM) acids. All analyses were performed with a flow rate of 1 mL/min and temperature of 25°C. Detection was at 282 nm and a 20μl sample size was used.

Dilution and Weight Errors

Dilution error was determined by analysis of three samples (200 mg/L neohesperidin DC, analysed six times) obtained from a 800 mg/L neohesperidin DC stock solution in DMSO. For weight error determination, four different amounts of neohesperidin DC (10, 20, 40 and 50 mg) were weighed three times each, and dissolved in DMSO until a final concentration of 200 mg/L. Analyses were carried out six times.

Straight Line Calibration

Three series (six samples each, at 100, 150, 200, 300, 400, and 500 mg/L neohesperidin DC dissolved in DMSO) were injected on three different days. Each sample was analysed six times. The final calibration straight line was taken as the mean of the slopes, independent of terms and correlation values of the three straight lines obtained, in accordance with reference 10.

Accuracy

Samples of neohesperidin DC were prepared in triplicate at 100, 150, 200, 300, 400, and 500 mg/L and were analysed six times. Accuracy was checked by the straight line obtained from the measured versus the theoretical neohesperidin DC concentrations, in accordance with reference 11.

Precision, Repeatability, and Reproducibility

The system precision was measured by the R.S.D. value of ten replicate injections of a 100 mg/L neohesperidin DC sample, according reference 11. The precision of the method was determined,[11] with five 100 mg/L neohesperidin DC samples analysed six times at the same session. Additionally, for repeatability determination, four 200 mg/L neohesperidin DC samples were analysed on four different days, six times every day. Reproducibility was tested, employing three different HPLC equipments, in each of which three 200 mg/L neohesperidin DC samples were analysed six times.

To ascertain the method precision, the repeatability and the reproducibility, R.S.D. values were determined; repeatability and reproducibility values were obtained.[12]

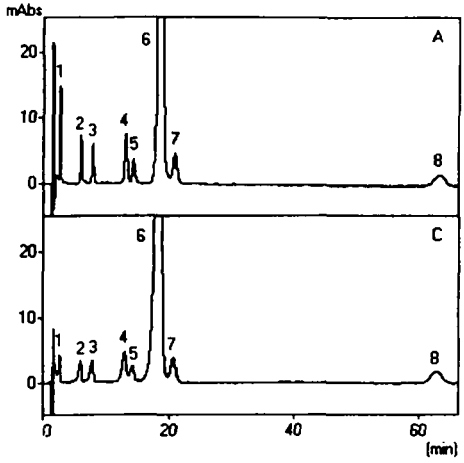

Figure 1. Chromatograms of samples A and C with 20% ACN and RP-C18 column as mobile and stationary phases, respectively, 1 mL/min flow rate, 25°C, 282 nm, 20μL sample size, 200 mg/L neohesperidine DC concentration and 4 mg/L other compounds concentration. The following compounds are present: FANH (1), NA (2), NH (3), NADC (4), HEDC (5), NHDC (6), HPDCG (7) and HPDC (8).

RESULTS AND DISCUSSION

Effects of Mobile Phase and of Sample Preparation Solvent

Of the different methanol:water and ACN:water elution systems assayed, 20% ACN gave the best results. Chromatograms of samples A and C eluted with 20% ACN are shown in Figure 1, and Table 1 reports the chromatographic parameters obtained from these analyses. Longer retained compounds showed higher than recommended k′ values (≈10),[9] because it was necessary to prolong the operation time to separate the eight different compounds, contained in samples A and C, some of them with very similar chromatographic behaviour. All α values were higher than the minimum accepted (1.05).[8] Resolution values were, in general, higher for sample A (with DMSO as solvent) than for sample C (with ACN as solvent).

This observation and the high As value (0.9-1.1 accepted range)[8] of sample C determined the selection of DMSO as standard solvent for sample preparation.

Table 1

Chromatographic Parameters Obtained for Samples A (with DMSO as Solvent) and C (with 50% ACN S solvent) Under Selected Standard Conditions

Compound	Sample A				Sample C			
	As	K′	α	Rs	As	K′	α	Rs
FANH	1.62	0.99			1.69	1.04		
			4.31	8.65			3.63	5.64
NA	1.07	4.28			2.38	3.78		
			1.21	3.86			1.43	2.96
NH	1.13	5.19			1.90	5.39		
			1.75	7.37			1.76	6.20
NADC	0.93	9.07			1.53	9.48		
			1.12	1.73			1.12	1.51
HEDC	1.00	10.15			1.44	10.61		
			1.33	4.39			1.32	3.98
NHDC	1.06	13.45			1.62	14.06		
			1.13	2.12			1.13	2.95
HPDCG	0.97	15.24			1.47	15.93		
			3.14	20.87			3.13	20.96
HPDC	1.02	47.86			1.00	49.88		

No differences were observed between acetic and phosphoric acids when used for mobile phase acidification. The selected conditions for neohesperidin DC analysis were, therefore, 20% ACN as mobile phase, acidified with 50 mM acetic acid, 1 mL/min flow rate, and neohesperidin DC dissolved in DMSO.

Optimisation of Sample Preparation.

It was necessary to use a neohesperidin DC concentration high enough to detect possible impurities present at the sample. Thus, 200 mg/L neohesperidin DC was selected as a standard concentration for sample preparation to allow detection of structurally related compounds. Dilution and weight errors were determined as reported in Materials and Methods. The R.S.D. for dilution error was 0.17%, thus making no significant contribution to the total error of the method.

Table 2

Linearity of Recovery Results for Neohesperidin DC Analysis

Assay Day	Added (mg/L)	Mean Found (n=6) ± SD (mg/L)	Recovery (%)	Mean Recovery (%)	R.S.D. Recovery (%)
1	98.9	98.6 ± 0.9	99.7		
2	95.8	95.1 ± 0.7	99.3	99.7	0.35
3	92.5	92.5 ± 1.2	100.0		
1	147.0	148.9 ± 1.2	101.3		
2	137.9	134.8 ± 1.0	97.7	99.2	1.87
3	139.7	137.9 ± 1.6	98.7		
1	182.4	181.9 ± 1.0	99.7		
2	181.5	180.0 ± 0.6	99.2	99.5	0.25
3	187.8	186.8 ± 0.3	99.5		
1	273.1	284.1 ± 3.3	104.0		
2	272.2	269.9 ± 1.2	99.2	101.7	2.36
3	274.0	278.8 ± 0.5	101.8		
1	368.4	376.2 ± 3.1	102.1		
2	363.8	361.4 ± 1.4	99.3	99.8	2.14
3	374.7	366.8 ± 2.9	97.9		
1	457.3	459.5 ± 3.1	100.5		
2	457.3	453.0 ± 2.5	99.1	99.8	0.70
3	460.0	458.8 ± 2.4	99.7		
		Mean:		99.9	1.28
		Slope:			0.9991
		Intercept:			0.48
		Correlation coefficient:			0.9998

The four amounts of neohesperidin DC assayed for weight error determination showed R.S.D. values ranging from 0.22% to 2.05%, the lowest values being for 50 mg of neohesperidin DC. Taking into consideration the best solvent as described above, the standard sample was prepared at 200 mg/L, by dissolving 50 mg of neohesperidin DC in 250 mL of DMSO.

Table 3

Method Precision of Neohesperidin DC Analysis

Sample (n°)	Mean Found (n=6) ± SD (mg/L)
1	89.9 ± 0.7
2	89.9 ± 0.3
3	89.9 ± 0.4
4	90.3 ± 0.4
5	90.6 ± 0.5
Mean ± SD (mg/L):	90.2 ± 0.3
R.S.D. (%):	0.33

Table 4
Repeatability of Neohesperidin DC Analysis

Sample (n°)	Mean Found (n=6) ± SD (mg/L)
1	188.3 ± 1.4
2	186.3 ± 0.9
3	184.7± 1.4
4	186.4 ± 2.0
Mean ± SD (mg/L):	186.4 ± 1.3
R.S.D. (%):	0.68
r (mg/L):	3.6

Validation Parameters

Linearity

The straight line obtained is expressed by the equation: Y= 34025 X - 1149 with r=0.9998, where X is neohesperidin DC concentration in mg/L and Y is the peak area. A linearity range 0-500 mg/L was demonstrated.

Table 5

Reproducibility of Neohesperidin DC Analysis

Equipment (n°)	Sample (n°)	Mean Found (n=6) ± SD (mg/L)	Mean (n=3) ± SD (mg/L)
	1	173.5 ± 0.4	
1	2	176.2 ± 1.1	175.5 ± 1.8
	3	176.8 ± 0.5	
	1	178.9 ± 0.6	
2	2	181.2 ± 1.6	180.0 ± 1.2
	3	179.8 ± 0.9	
	1	178.8 ± 0.5	
3	2	176.9 ± 1.0	177.7 ± 1.0
	3	177.6 ± 0.8	
	Mean ± SD (mg/L):		177.7 ± 1.8
	R.S.D. (%):		1.03
	R (mg/L):		5.1

Accuracy

The accuracy of the described method is shown by the linearity of the recovery data for the neohesperidin DC analyses.[11] The results are shown in Table 2. The slope of the straight line obtained from the theoretical vs the measured data was 0.9991. The highest R.S.D. was 2.36% and mean recovery was 99.9%, with an acceptable mean R.S.D. of 1.28%;

Precision, Repeatability, and Reproducibility

The precision of the system was acceptable with an R.S.D. of 0.74%. The results shown in Table 3 reveal the high precision of the method with an R.S.D. of 0.33% between the different samples. Additionally, repeatability and reproducibility were also determined.

The results are shown in Tables 4 and 5, respectively. R.S.D. values were 0.68% in determinations performed on different days, and 1.03% between the three HPLC equipments assayed. Repeatability and reproducibility values were 3.57 and 5.12 mg/L, respectively.

Selectivity

In previous research, neohesperidin DC was purified by preparative HPLC, and its structure confirmed by RMN, IR and MS analyses (unpublished results). Purified neohesperidin DC (99.9% chromatographic purity) was employed as an external standard to check the HPLC profile of commercial neohesperidin DC (L29024). The same retention time (17.4 min) was observed for both purified and commercial neohesperidin DC samples. No analytical interferences between neohesperidin DC and the added related compounds was detected. Resolution values were above the minimum recommended (Rs > 1.5, Table 1).[8] The analytical method used could therefore, be considered specific for checking the purity of neohesperidin DC.

CONCLUSIONS

In conclusion, this HPLC method is suitable and selective for neohesperidin DC analysis, regardless of the equipment used and laboratory conditions. The method shows very high accuracy, precision and repeatability values.

REFERENCES

1. R. M. Horowitz, B. Gentilli, "Dihydrochalcone Sweeteners from Citrus Flavanones" in **Alternative Sweeteners**, L. O'Brien, R. C. Gelardi, eds., Marcel Dekker, Inc. New York, 1991, pp. 97-115.

2. M. G. Lindley, I. Canales, F. Borrego, FIE 91 Conference Proceedings, Paris, Expoconsult, Maarsen, 1991, pp. 48-51.

3. F. Borrego, I. Canales, M. G. Lindley, Z. Lebens. Unters. Forsch, **200**, 32-37 (1995).

4. M. G. Lindley, P. K. Beyts, I. Canales, F. Borrego, J. Food Sci., **58**, 592-596 (1993).

5. Anonymous, European Parliament and Council Directive 94/35/, Off. J. Eur. Comm., September 16, N° C 237/12 (1994).

6. Anonymous, European Parliament and Council Directive 95/2/EC, Off. J. Eur. Comm., March 18, N° L 61/1 (1995).

7. Anonymous, Commission Directive 95/31/EC, Off. J. Eur. Comm., July 28, N° L 178/1 (1995).

8. L. R. Snyder, J. L. Glajch, J. J. Kirkland, **Practical HPLC Method Development**, John Wiley & Sons Inc. New York, 1988.

9. S. Lindsay, **High Performance Liquid Chromatography**, John Wiley & Sons Ltd., Chichester, 1992.

10. M. A. Camacho, A. I. Torres, M. E. Gil, M. M. Obregón, Tecnifarma, **4**, 10-12 (1993).

11. T. D. Wilson, W. G. Jump, W. C. Newmann, T. San Martín, J. Chromatogr., **641**, 241-248 (1993).

12. Anonymous, J. Assoc. Off. Anal. Chem., **71**, 161-173 (1988).

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