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Comparison of methods for determining coumarins in distilled beverages

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Abstract

Two analytical methods, high performance liquid chromatography and spectrofluorimetry, were studied to determine the content of coumarins (umbelliferone, scopoletin and 4-methylumbelliferone, in distilled beverages). Hydro-alcohol standard solutions of known coumarin concentration and commercial white rum samples were used to compare them. After determining the coumarin content with both methods and performing a statistical analysis of the results obtained, the conclusion was reached that although both techniques are valid for this purpose, the spectrofluorimetric method is more accurate than high performance liquid chromatography.

Keywords: HPLC; Spectrofluorimetry; Coumarins; Umbelliferone; Scopoletin, 4-Methylumbelliferone

1. Introduction

The most notable physical characteristic of the majority of natural coumarins is the fact that they are fluorescent in UV light (Evans, 1991; Murray, et al., 1982).

Various analytical techniques based on this property have been developed, such as thin-layer chromatography on silica-gel slides (Abu-mustafa et al., 1969; Martelli & Calvino, 1971) or on silica-gel cellulose slides, to determine levels of coumarins in phenolic plants using UV light and the diazo reagents, *p*-nitroaniline and sulphanilic acid, with different mixtures of these solvents (Greninger, 1970).

The Association of Official Analytical Chemists (AOAC, 1990) recommended gas-phase chromatography as the official analytical method to determine coumarins in wine. This method was previously used by Quercia (1968) to quantify the small amounts of coumarins present in mixtures obtained from plant extracts, such as umbelliferone and scopoletin.

Nowadays, experienced authors in the field of enological research such as Puech and Moutounet (1988) and Salagoity et al. (1987), favour the use of high performance liquid chromatography (HPLC) with a UV detector to determine the levels of coumarins, both in hydro-alcohol solutions, in oak barrels and in distilled alcoholic beverages.

Photochemical and spectroscopic studies of coumarins and coumarin derivatives have been carried out (Bazyl et al., 1998; Chen & Chou, 1995; Chen & Jean, 1997; Gallivan, 1977; Shim & Kyung, 1976). The latter study found fluorescence to be the main emission by coumarins in non-polar solvents, which is different from the fluorescence emitted by coumarins when they are located in polar solvents.

On the other hand, the determination of coumarins in alcoholic beverages by spectroscopic techniques has only been described by Otsuka and Zenibayashi (1974), who applied a fractioning technique to the compounds, which enabled them to quantify the scopoletin, the only coumarin found in the samples of alcoholic beverages analysed, by fluorescence. This study revealed the dependence of fluorescence on environmental factors, such as the pH, and polar solvents. Fluorescence, therefore, is very useful for the localization and recovery of coumarins marked in a chromatogram without having to use a chemical spray (Murray et al., 1982).

Despite the various analytical techniques available for the determination of coumarins, there is a persistent

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Table 1
Mean coumarin concentrations foound by HPLC and spectrofluorimetry for each of the 60%v/v hydro-alchol standard solutions[a]

Coumarin mean concentration found by HPLC ($n=10$)			Real	Coumarin mean concentration found by spectroflurimetry ($n=10$)		
A (μg/l)	B (μg/l)	C (μg/l)	(μg/l)	A (μg/l)	B (μg/l)	C (μg/l)
7.98 ± 0.34	8.06 ± 0.28	6.01 ± 0.12	15.0	17.2 ± 0.65	16.0 ± 0.23	16.2 ± 0.21
20.1 ± 0.27	16.1 ± 0.54	20.0 ± 0.29	20.0	30.9 ± 0.55	33.1 ± 0.62	32.1 ± 0.37
40.1 ± 0.39	40.1 ± 0.23	40.0 ± 0.14	40.0	50.1 ± 0.63	54.1 ± 0.45	51.5 ± 0.56
79.8 ± 0.16	80.1 ± 0.23	80.0 ± 0.44	80.0	101 ± 0.78	94.9 ± 0.29	98.3 ± 0.25
120 ± 0.37	119 ± 0.21	120 ± 0.35	120.0	128 ± 1.69	124 ± 0.19	125 ± 0.16
160 ± 0.25	160 ± 0.45	160 ± 0.51	160.0	155 ± 0.35	150 ± 0.21	152 ± 0.39
200 ± 0.30	200 ± 0.70	316 ± 0.27	200.0	180 ± 0.43	186 ± 0.33	176 ± 0.43
286 ± c0.35	240 ± 0.55	352 ± 0.38	240.0	247 ± 0.80	241 ± 0.48	241 ± 0.69
300 ± 0.45	418 ± 1.20	421 ± 0.25	280.0	274 ± 0.78	268 ± 0.28	268 ± 0.50

[a] A, scopoletin; B, umbelliferone; C, 4-methylumbelliferone.

problem because of the very small quantities of coumarins that are usually found in alcoholic beverages and the varying resolution and efficiency of existing analytical techniques. Moreover, there is not yet any officially-stipulated method for the determination and quantification of the coumarins under study, whether in alcoholic beverages or any other foodstuff, that might serve as a reference. Hence the current study is justified.

As we wish to find a single, fast and, above all, reliable analytical method to determine levels of coumarins, it was decided to compare high performance liquid chromatography (HPLC) with another analytical technique, spectrofluorimetry, to reach conclusions regarding an efficient method to quantify the levels of coumarins in distilled beverages.

2. Materials and methods

2.1. Samples analysed

The two methods were compared by means of hydro-alcohol standard solutions of 60% v/v (distilled-deionized water and analytical-grade Panreac alcohol were used) which is the strength at which this kind of alcoholic beverage is aged. The standard solutions contained the following quantities of umbelliferone, scopoletin and 4-methylumbelliferone (all obtained from Sigma): 15, 20, 40, 80, 120, 160, 200, 240 and 280 μg/l, thus constituting the model system (Table 1). Three samples of a distilled beverage (a commercially-available brand of white rum) were also used. Both methods (HPLC and spectrofluorimetry) were applied to determine the concentration of scopoletin, umbelliferone and 4-methylumbelliferone in these samples (Fernández, 1998) (Table 2). These samples also comprised the basis for comparison of the two methods to determine coumarins in distilled beverages.

2.2. Analytical method

2.2.1. Sample preparation

All the samples, both for analysis by HPLC and by Spectrofluorimetry (FLMT), were previously submitted to a process of component extraction, using the technique described by Salagoity et al. (1987).

2.2.2. High performance liquid chromatography

The determination of coumarins by HPLC was performed under the following chromatographic conditions (Fernández, 1998):

Mobile phase (A), with double distilled water containing 3% glacial acetic acid; (B), acetonitrile with 3% glacial acetic acid; solvent flow, 1 ml/min. The elution gradient to obtain correct separation of the coumarins was: at time 0 min, 94% (A) and 6% (B); at time 10 min, 94% (A) and 6% (B); at time 30 min, 82% (A) and 18% (B); at time 35 min, 67% (A) and 33% (B); at time 40 min, 42% (A) and 58% (B). With this gradient, the spike corresponding to scopoletin appeared at 25 min, the spike corresponding to umbelliferone at 20 min and to 4-methylumbelliferone, at 29 min.

Chromatographic separation of the coumarins was obtained using a Spherisorb S5 ODS-2 inverse phase CH-18 column, 20 cm long and with an internal diameter of 4.6 mm.

Table 2
Coumarin concentrations found by HPLC and spectrofluorimetry in commercial white rum samples[a]

HPLC				Spectrofluorimetry			
Samples	A (μg/l)	B (μg/l)	C (μg/l)	C (μg/l)	B (μg/l)	A (μg/l)	Samples
1	0	0	0	0	0	0	1
2	213 ± 0.87	0	0	0	0	216 ± 0.76	2
3	381 ± 1.05	0	0	0	0	387 ± 0.88	3

[a] A, scopoletin; B, umbelliferone; C, 4-methylumbelliferone.

The coumarins were detected using a fluorescence detector with excitation and emission wavelengths of 340 and 425 nm, respectively. A volume of 15 μl of each sample was injected into the chromatograph at room temperature.

2.2.3. Spectrofluorimetry

The spectrofluorimetric study was carried out with a Shimadzu RF-5.001PC spectrofluorimeter, to determine levels of fluorescence in the coumarins in a stationary state. The light source used was a Xenon 150 w lamp with an optical system composed of two automatic monochromators, one for excitation and the other for emission, of a mesh type to enable a suitably wide selection of excitation and emission wavelengths for the coumarins. The optimum excitation and emission wavelengths for coumarins are 340 and 425 nm, respectively (Otsuka & Zenibayashi, 1974). A quartz cell was used (Ira, 1993). The detection system comprised a R450-01 photomultiplier which transformed the fluorescent radiation emitted by the scopoletin solution in the cell into an electrical signal. The thermostat system used was a Braun Frigomix 1.450B with a water-recycling system for temperature control, which for our purposes was fixed at $25 \pm 1^{\circ}C$. Finally, the spectrofluorimetric tests were carried out with variable-width excitation and emission slits set at an aperture of 3.0 nm.

2.2.4. Statistical tests

All the statistical tests described in this paper were performed using STSC STATGRAPHICS PLUS 2.0 and SPSS SYSTAT 7.0 statistical analysis software.

3. Results and discussion

The study was carried out using the two sample types described above:

1. Model system
2. Commercially-available white rum

3.1. Study of the model system

Table 1 describes the mean concentrations of scopoletin, umbelliferone and 4-methylumbelliferone, determined by 10 measurements of each hydro-alcohol standard solution, using HPLC and spectrofluorimetry techniques.

The statistical method proposed by Martin and Luna del Castillo (1990) was used to compare the accuracy of the two techniques. This method is based on the choice of a hydro-alcohol standard solution that is as close as possible to the mean concentration of the concentrations being studied, which in this case was 120 μg/l. Taking into account the arithmetic mean of the 10 measurements obtained for each concentration, the accuracy is expressed as a percentage recovery rate. Other parameters determined for each analytical technique were the relative error, the standard deviation, the standard error or mean deviation and the coefficient of variation (Tables 3–5).

The statistical study reveals that the HPLC method presents a better recovery rate, in all cases close to 100%. The spectrofluorimetry technique, however, although close to the former results, in every case

Table 3
Statistical treatment proposed by Martin and Luna del Castillo (1990) for 60% v/v hydro-alcohol standard solutions of spectrofluorimetry

	HPLC	Spectrofluorimetry
Real concentration (μg/l)	120.0	120.0
Mean concentration X_m (μg/l)	120.0	128.0
Recovery (%)	100.0	107.0
Standard deviation (S.D.)	0.37	1.70
Variation coefficient (V.C.)(%)	0.31	1.33
Standard error (S.M.)	0.12	0.54
Relative error (R.E.)	0.23	0.95
$X_m + S_m \times t$	121.0	129.0
$X_m - S_m \times t$	120.0	127.0

Table 4
Statistical treatment proposed by Martin and Luna del Castillo (1990) for 60% v/v hydro-alcohol standard solutions of umbelliferone, using HPLC and spectrofluorimetry

	HPLC	Spectrofluorimetry
Real concentration (μg/l)	120.0	120.0
Mean concentration X_m (μg/l)	119.0	124.0
Recovery (%)	99.2	103.0
Standard deviation (S.D.)	0.21	0.19
Variation coefficient (V17.C.)(%)	0.18	0.16
Standard error (S.M.)	0.07	0.06
Relative error (R.E.)	0.13	0.11
$X_m + S_m \times t$	119.0	124.0
$X_m - S_m \times t$	119.0	124.0

Table 5
Statistical treatment proposed by Martin and Luna del castillo (1990) for 60% v/v hydro-alcohol standard solutions of 4-methylumbelliferone, using HPLC and spectrofluorimetry

	HPLC	Spectrofluorimetry
Real concentration (μg/l)	120.0	120.0
Mean concentration X_m (μg/l)	120.0	125.0
Recovery (%)	100.0	105.0
Standard deviation (S.D.)	0.35	0.16
Variation coefficient (V.C.)(%)	0.29	0.13
Standard error (S.M.)	0.11	0.05
Relative error (R.E.)	0.21	0.09
$X_m + S_m \times t$	120.0	126.0
$X_m - S_m \times t$	120.0	125.0

produced a recovery rate in excess of 100%. As this parameter was taken as a measure of the accuracy of the technique, the conclusion is reached that, although the differences between the results obtained by the two techniques are minimal, HPLC presents a greater level of accuracy than spectrofluorimetry, according to the criteria of Martin and Luna del Castillo (1990). Nevertheless, on considering the values of standard deviation, coefficient of variation and relative error, all of which are parameters that describe the precision of a technique, we see that these are, in fact, better for the spectrofluorimetry technique. Thus, the latter is virtually as accurate as HPLC but provides a higher degree of precision.

The results obtained thus far were not conclusive, being based only on the study of a hydro-alcohol standard solution of moderate concentration. It was necessary to confirm them by considering all the standard solutions available, and by using other statistical methods to test whether the previous results were repeated or whether significant differences regarding the accuracy of the two analytical methods were found.

Firstly, and with this in mind, a correlation analysis was carried out using the real concentrations of scopoletin, umbelliferone and 4-methylumbelliferone present in the standard solutions used, in conjunction with the mean concentrations found by each of the two analytical techniques (Quesada et al., 1995). The results of this study are summarised in Table 6, where it is evident that the correlation coefficients for spectrofluorimetry are clearly higher than those obtained with HPLC. For example, both the regression coefficients found for umbelliferone and for 4-methylumbelliferone are much higher than the respective coefficients obtained with HPLC, which indicates that the concentrations of these coumarins, as found by spectrofluorimetry, are closer to the real levels than the results obtained with HPLC, taking into account that the closer the correlation coefficient is to 1.000, the greater is the similarity between the concentrations being compared. Therefore, for these two coumarins, and according to the correlation analysis performed, the spectrofluorimetry technique is more accurate than HPLC. For scopoletin, however, the coefficients obtained by the two techniques are identical; in the case of this coumarin, therefore, it is not possible to state that either technique is better than the other.

Secondly, a two-sample comparison test was performed, as proposed by Porretta and Sandei (1991) and by Quesada et al. (1995). To do this, and in every case before carrying out the comparison test, the type of distribution present in the sample populations under study was determined, together with the results obtained by HPLC and by spectrofluorimetry and the theoretical concentrations of the coumarins used. Application of the Kolmogorov–Smirnov test for goodness-of-fit in a normal distribution showed that all the sample populations presented a normal distribution (Table 7), and so a parametric test to compare two samples, the *t*-test, could be used.

Comparison of the sample populations by the *t*-test did not reveal any significant statistical differences between the coumarin concentrations found by HPLC and by spectrofluorimetry. In every case, the level of significance was $P < 0.05$ (Table 8). However, the levels of significance obtained by the *t*-test were identical to those found in the correlation study. Thus, for umbelliferone and 4-methylumbelliferone, levels of significance were higher with spectrofluorimetry than those found with HPLC, which again demonstrates that the spectrofluorimetry technique, for these two coumarins in particular, is more accurate than HPLC. In the case of scopoletin, on the other hand, a greater difference with respect to the results obtained in the regression analysis is obtained. In the latter analysis, regression coefficients

Table 6
Correlation coefficients obtained from the correlation analysis between the concentrations of coumarins found by HPLC and spectrofluorimetry and the real concentrations present in the standard solutions

	HPLC	Spectrofluorimetry
Correlation coefficients	Correlation coefficients	Correlation coefficients
Scopoletin-real	0.994	0.994
Umbelliferone-real	0.960	0.996
4-methylumbelliferone-real	0.982	0.994

Table 7
Type of distribution of the samples populations in the model system

Kolmogorov–Smirnov test at 95% confidence		
Sample populations	*P*-value	Distribution
Scopoletin by HPLC	0.990	Normal
Umbelliferone by HPLC	0.990	Normal
4-methylumbelliferone by HPLC	0.470	Normal
Scopoletin by spectrofluorimetry	0.990	Normal
Umbelliferone by soectrofluorimetry	0.990	Normal
4-methylumbelliferone by spectrofluorimetry	0.990	Normal
Real standard contents	0.990	Normal

Table 8
Levels of significance found by application of the *t*-test

t-test at 95% confidence		
Comparison	HPLC	Spectrofluorimetry
	P-value	*P*-value
Scopoletin-real	0.890	0.940
Umbelliferone-real	0.800	0.970
4-Methylumbelliferone-real	0.520	0.980

were identical for both techniques, while the *t*-test confirmed the tendency observed in the other two coumarins, i.e. a greater level of significance was obtained for spectrofluorimetry than for chromatography. Therefore, at present and in the light of these results, the fluorimetric technique must be considered more accurate than HPLC (Figs. 1–3).

3.2. Study of commercially-available white rum samples

As in the previous section, a comparative study of the accuracy of high performance liquid chromatography and spectrofluorimetry techniques was carried out, but in this case using three samples of commercially-available white rum, after previous determination of the content of scopoletin, umbelliferone and 4-methylumbelliferone (Table 2).

Standard quantities of the rum samples, with a known concentration of the three coumarins, were then obtained. Four aliquots were taken from each of the samples, and 80 μg/l of scopoletin, umbelliferone and 4-methylumbelliferone were added to two of them, and 160 μg/l to the other two (Quesada et al., 1995).

The coumarin content of the solutions prepared was determined using the analytical techniques described above, with accuracy again being expressed as the percentage recovery rate (Table 9).

The recovery rates found after adding the 80 and 160 μg/l coumarin solutions again show the higher accuracy presented by the spectrofluorimetric technique with respect to chromatography. As in the model system, however, the differences were minimal. The difference, in this case, is the fact that the recovery rates obtained by spectrofluorimetry for the three coumarins are always less than 100%, which was not the case for the model system, although the rates are still higher than those obtained by HPLC.

Secondly, and as in the model system, a correlation study was carried out between the real concentrations of coumarins present in the samples of white rum and those found after applying the two techniques (Table 10). The study showed a good correlation in every case, and in particular that obtained by spectrofluorimetry for scopoletin ($r = 1.000$). Nevertheless, in the case of umbelliferone, by spectrofluorimetry, a lower correlation index was obtained than for the model system. Thus, in this particular case, the chromatographic technique was found to be more accurate than spectrofluorimetry, unlike the other two coumarins, which, as in the model system, presented very similar correlation indices.

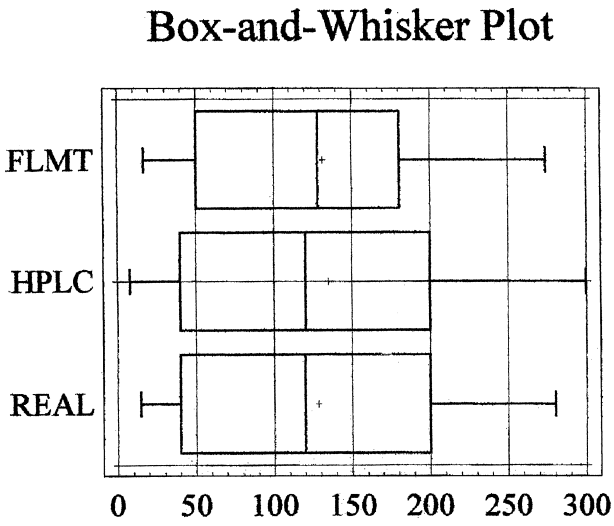

Fig. 1. Box-and-Whisker plot of the *t*-test for scopoletin in the model system.

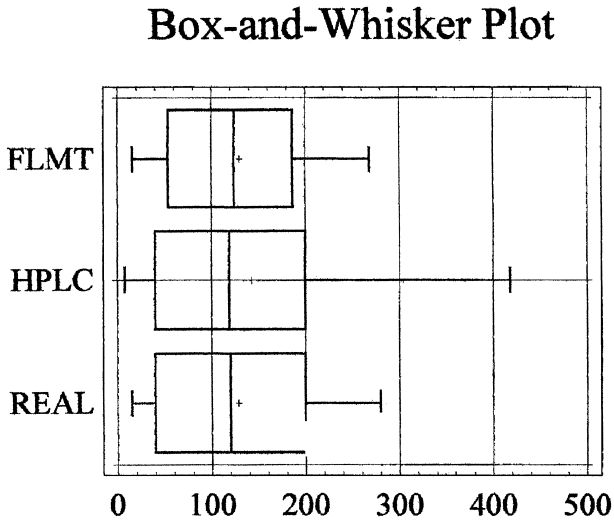

Fig. 2. Box-and-Whisker plot of the *t*-test for umbelliferone in the model system.

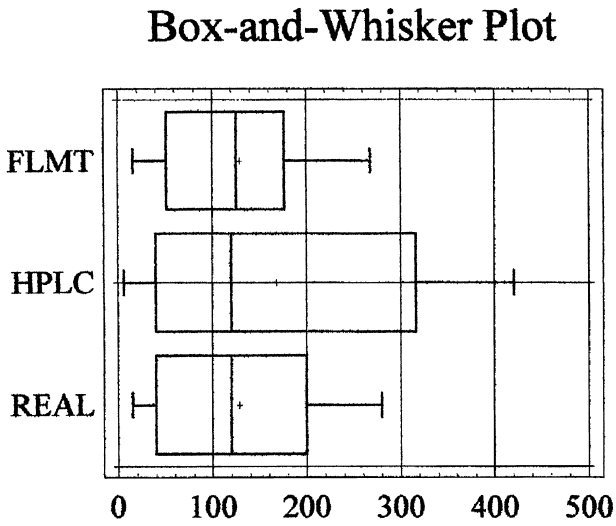

Fig. 3. Box-and-whisker plot of the *t*-test for 4-methylumbelliferone in the model system.

To conclude this study of the white rum samples, a comparison was made between the sample populations and their real concentrations. Firstly, the Kolmogorov–Smirnov test was applied to the sample populations studied, in order to determine whether they presented a normal distribution and thus a *t*-test could be used or whether, on the contrary, a non-parametric test such as the Wilcoxon test would be required (Table 11). The results of the Wilcoxon test are given in Table 12, which shows there are no statistically significant differences between the coumarin concentrations determined by the two techniques and the real concentrations present in the samples of white rum. Nevertheless, as in the previous correlation study, better levels of significance were observed for the determination of umbelliferone by HPLC than by spectrofluorimetry, while the other coumarins continued the trend established in the earlier tests, i.e. better levels of significance, though with only minimal differences, and better recoveries both in the model system and for the samples of white rum.

Table 9
Recovery (%) of coumarins in commercial white rum samples

HPLC						Spectrofluorimetry					
Samples	Initial amount (μg/l)	Added (μg/l)	Total (μg/l)	Found (μg/l)	Rec. (%)	Rec (%)	Found (μg/l)	Total (μg/l)	Added (μg/l)	Initial amount (μg/l)	Samples
Scopoletin											
1	0	80	80	74 ± 0.54	92	96	77 ± 0.25	80	80	0	1
		160	160	148 ± 0.76	92	97	155 ± 0.48	160	160		
2	213 ± 0.87	80	293	240 ± 0.79	82	95	281 ± 0.63	296	80	216 ± 0.76	2
		160	373	313 ± 0.86	84	97	365 ± 0.56	376	160		
3	381 ± 1.05	80	461	400 ± 1.12	87	94	439 ± 0.68	467	80	387 ± 0.88	3
		160	541	476 ± 0.55	88	96	525 ± 0.72	547	160		
		Mean recovery = 87.5%				Mean recovery = 95.8%					
Umebeliferone											
1	0	80	80	74 ± 0.26	92	97	78 ± 0.23	80	80	0	1
		160	160	148 ± 0.65	92	97	155 ± 0.56	160	160		
2	0	80	80	73 ± 0.31	91	95	76 ± 0.32	80	80	0	2
		160	160	147 ± 0.75	92	97	155 ± 0.41	160	160		
3	0	80	80	76 ± 0.29	95	95	76 ± 0.32	80	80	0	3
		160	160	150 ± 0.80	94	98	157 ± 0.81	160	160		
		Mean recovery = 92.6%				Mean recovery = 96.5%					
4-Methylumbelliferone											
1	0	80	80	78 ± 0.35	97	97	78 ± 0.45	80	80	0	1
		160	160	158 ± 0.66	99	99	158 ± 0.97	160	160		
2	0	80	80	77 ± 0.42	96	95	76 ± 0.46	80	80	0	2
		160	160	154 ± 085	96	95	152 ± 0.87	160	160		
3	0	80	80	72 ± 0.42	94	96	77 ± 0.65	80	80	0	3
		160	160	153 ± 0.75	96	98	157 ± 0.89	160	160		
		Mean recovery = 95.3%				Mean recovery = 96.6%					

Table 10
Correlation coefficients obtained from the correlation analysis between the concentrations of coumains found by HPLC and spectrofluorimetry and the real concentrations present in commercial white rum samples

Pearson correlation analysis		
Comparison	HPLC *P*-value	Spectrofluorimetry *P*-value
Scopoletin-real scopoletin	0.998	1.000
Umbelliferone-real umbelliferone	0.999	0.714
4-Methylumbelliferone-real 4-methylumbelliferone	0.999	0.999

Table 11
Type of distribution of the sample populations of commercial white rum samples

Kolmogorov–Smiromov test at 95% confidence		
Sample populations	*P*-values	Distribution
Scopoletin by HPLC	0.997	Normal
Umbelliferone by HPLC	$P < 0.05$	Not normal
4-Merthylumbelliferone by HPLC	$P < 0.05$	Not normal
Scopoletin by spectrofluorimetry	0.999	Normal
Umbelliferone by spectrofluorimetry	$P < 0.05$	Not normal
4-Methylumbelliferone by spectrofluorimetry	$P < 0.05$	Not normal
Real standard contents of scopoletin by HPLC	0.999	Normal
Real standard contents of umbelliferone by HPLC	$P < 0.05$	Not normal
Real standard contents of 4-methylumbelliferone by HPLC	$P < 0.05$	Not normal
Real standard contents of scopoletin by spectrofluorimetry	0.998	Normal
Real standard contents of umbelliferone by spectrofluorimetry	$P < 0.05$	Not normal
Real standard contents of 4-methylumbelliferone by spectrofluorimetry	$P < 0.05$	Not normal

Table 12
Levels of significance found by application of the *t*-test and Wilcoxon test to commercial white rum samples

t-*test*		
	HPLC	Spectrofluorimetry
Comparison	*P*-value	*P*-vlaue
Scopoletion-real scopoletin	0.661	0.892
Wilcoxon test at 95% confidence		
Umbelliferone-real umbelliferone	0.729	0.508
4-Methylumbelliferone-real 4-methylumbelliferone	0.871	0.888

4. Conclusion

To sum up, the results obtained demonstrate the appropriateness of both spectrofluorimetry and high performance liquid chromatography to determine the level of coumarins in distilled beverages. Nevertheless, the two techniques can be distinguished in terms of the accuracy they provide. The HPLC technique is shown, by the statistical treatment applied, based on the recovery rate method, to be accurate but less precise than spectrofluorimetry. The latter, according to the results obtained from the other statistical tests, both in the model system and with the samples of white rum, is found to be a technique that is somewhat more accurate than HPLC, under the chromatographic conditions described in this study. However, it might be possible to vary these chromatographic conditions and improve the precision of the HPLC technique, thus equalling the performance of spectrofluorimetry in this respect. Furthermore, the ease of use and speed of analysis of HPLC give it an advantage with respect to spectrofluorimetry, and so the analyst must choose between two techniques, which are both perfectly valid to determine levels of coumarins, on the sole basis of the requirements of accuracy and speed of application.

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