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Quantification in Comprehensive Two-Dimensional Liquid Chromatography

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Although the use of comprehensive two-dimensional liquid chromatography (LC×LC) as a powerful separation technique is continuously increasing, its employment in quantification experiments is rather limited. The present research is focused on the quantification of a series of standards, as well as real-world sample compounds, by using dedicated laboratory-constructed LC×LC software, developed through a novel approach. Moreover, the difficulties encountered during software operation, in various elution conditions, are described and discussed. The results attained were compared with those observed in conventional LC, and no statistically significant differences were observed in the determination of aurapten in grapefruit oil. However, a loss in sensitivity was observed when using LC×LC (limit of detection = 0.10 ppm) compared to conventional LC (limit of detection = 0.05 ppm) as a consequence of the sample dilution in comprehensive two-dimensional liquid chromatography.

In recent years, the use of comprehensive two-dimensional liquid chromatography (LC×LC) has experienced a substantial increase. This technique, introduced 30 years ago by Erni and Frei[1] and further developed by Bushey and Jorgenson,[2] is characterized by a much greater resolving power compared to monodimensional LC. LC×LC systems enable the analysis of the entire initial sample in two different dimensions, coupled in-line by means of a transfer device, usually a 10-port switching valve. On the contrary, heart-cutting multidimensional approaches enable the second-dimension analysis of only a limited number of first-dimension fractions.[3] Theoretically, distinct separation methodologies can be employed (reversed phase, normal phase, size exclusion chromatography, ion exchange, etc.) in each dimension although, in practice, a series of problems can be encountered such as mobile phase immiscibility or first-dimension mobile phase and second-dimension stationary phase incompatibility. However, the combination of orthogonal separation mechanisms is preferred since the peak capacity enhancement, in this way, is maximized.[4–6]

Probably, the normal plus reversed-phase setup with a high degree of orthogonality is the most common LC×LC combination used.[7] However, a major problem related to such an approach is the poor second-dimension resolution, which is partially due to solvent immiscibility. In the present research, this drawback was overcome by using a microbore column and a conventional monolithic column in the first and second dimension, respectively. The employment of these columns produced a fundamental LC×LC characteristic, viz., a slow first-dimension separation and high-speed second-dimension analyses. Finally, the use of a secondary monolithic column minimizes back-pressure, a factor which could be, otherwise, too high to ensure fast-enough separations.

Nowadays, a good number of applications using LC×LC have been reported,[7] not only for proteomics[8–10] but also for the separation of polymers,[11–14] as well as other compounds;[15–20]

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however, quantification experiments have been rarely described.[21,22] In these applications, LC×LC−MS extracted ion chromatograms were exploited for the quantification process, which was carried out manually: peak areas relative to the same compound in different modulation windows were summed.

The lack of quantitative applications in LC×LC separations can be partially due to the fact that some parameters could require a different interpretation compared to that used for conventional monodimensional separations,[23] as well as the lack of dedicated software capable of identifying and quantifying more precisely a peak taken from a two-dimensional (2D) plot. In the present research, a series of pure standard and citrus-fruit compounds have been quantified by using LC×LC. The quantification process was achieved through an in-laboratory developed software that enabled the automatic and correct integration of each 2D peak. To the best of our knowledge, no previously published LC×LC-UV (or diode array detector (DAD)) works have involved automated quantification processes. Moreover, a series of quantitation aspects, such as the use of an internal standard and the effects of background subtraction, are considered. Finally, the LC×LC results attained are directly compared to those observed in a conventional LC application.

EXPERIMENTAL SECTION

Samples and Chemicals. Ethyl acetate and ethyl alcohol were purchased from Carlo Erba Reagenti (Milan, Italy); water, acetonitrile, and *n*-hexane were from VWR International (Milan, Italy). All solvents were HPLC grade.

Aurapten standard (7-geranyloxycoumarin) was isolated in the laboratory from grapefruit essential oil.[16] Coumarin and hexylbenzene were purchased from Fluka Chemie AG (Buchs, Switzerland), whereas toluene was provided by Acros Organics (New Jersey) and α-amylcinnamaldehyde, cinnamaldehyde, and cinnamyl alcohol by Sigma-Aldrich (Milan, Italy).

The orange and grapefruit essential oils were donated by a local producer.

Sample Preparation. *Aurapten Quantitation.* The analyses of aurapten and coumarin were performed using samples prepared/diluted in a mixture of *n*-hexane/ethyl acetate (4:1). For preparation of calibration curves, stock solutions for both compounds (1000 ppm) were prepared, from which further dilutions with different concentrations of aurapten (5−200 ppm) were derived. Coumarin was added as internal standard to all solutions at a concentration of 100 ppm. Aurapten was quantified in a standard solution and in a spiked orange essential oil. The grapefruit essential oil sample was diluted 1:100 with *n*-hexane/ethyl acetate (4:1) and spiked with an internal standard (coumarin) to a final concentration of 100 ppm.

Quantitative Analyses of the Standard Test Mixture. The analyses of mixtures of standards were performed using samples prepared/diluted in a mixture of *n*-hexane/ethanol (98:2). For calibration curve construction, stock solutions for all compounds (≅1000 ppm) were prepared, from which further dilutions with different concentrations of all compounds (ca. 5−500 ppm) were derived.

Instrumentation and Software. *LC Analyses of Aurapten.* The LC analyses were performed on a Shimadzu LC system (Shimadzu, Milan, Italy), consisting of an SCL-10Avp controller, a GC-17A gas chromatographic oven, two LC-10 ADVp pumps, a DGU-14A degasser, and an SPD-10Avp DAD detector. For data acquisition, class VP-5 software (Shimadzu) was used. The acquired data were converted and elaborated using LCsolution version 1.21 SP1 software (Shimadzu).

LC×LC Analyses of Aurapten and Standard Mixture. LC×LC analyses were performed on a Shimadzu Prominence LC2010^{square} LC×LC system (Shimadzu, Milan, Italy), consisting of a CBM-20A controller, an LC-20 AD and LC-20 ADSP dual-plunger parallel-flow pumps (LC 1), an LC-20 AB solvent delivery module equipped with two dual-plunger tandem-flow pumps (LC 2), a DGU-20A5 degasser, an SPD-20A UV detector, a CTO-20AC column oven, and an SIL-20AC autosampler. The two dimensions were connected by using an electronically controlled two-position 10-port Supelpro switching valve (Supelco, Milan, Italy), placed inside the column oven and equipped with two sample loops of equal volume. In the first dimension a Supelcosil LC-SI, 300 mm × 1.0 mm i.d., 5 μm dp (Supelco) column was used, while a Chromolith Flash C_{18}, 25 mm × 4.6 mm i.d. column, with a Chromolith guard column, 5 mm × 4.6 mm i.d. (Merck, Darmstadt, Germany) was employed in the second dimension. Both dimensions and the switching valve were controlled by the LCsolution version 1.21 SP1 software (Shimadzu). With regards to LC×LC data, the ASCII data files were created by using the export function of the LCsolution software, and this was converted and visualized by Chrom^{square} ver. 1.0 software (Chromaleont, Messina, Italy, a spin-off company of the University of Messina). The peaks were integrated and quantified by using the same in-laboratory constructed software.

Experimental Conditions. *LC Analyses of Aurapten.* The chromatographic column used for the conventional HPLC analyses was a Discovery HS C_{18} 150 mm × 4.6 mm i.d., 3 μm (Supelco). As mobile phase, water (A) and acetonitrile (B) were used in the following gradient conditions: 0−1 min 30% B, 1−5 min from 30% to 60% B, 5−8 min 60% B, 8−30 min from 60% to 100% B, 30−35 min from 100% to 30% B, 30−40 min 30% B.

An HPLC oven was used to maintain the column temperature at 30 °C, flow rate was 1 mL/min, and the 5 μL injections were made by a Rheodyne 7725i manual injector. The photodiode array detector was operated in 190−370 nm range, and the chromatograms were extracted at 315 nm.

LC×LC Analyses of Aurapten. The first-dimension mobile phase was composed of *n*-hexane and ethyl acetate (80:20), employed in isocratic elution. The flow rate was 18 μL/min, and the 2 μL injections were made by the autosampler.

In the second dimension acetonitrile (A) and water (B) were used as mobile phases, following a 1.1 min gradient: 0.2 min 70% B, 0.2−0.8 min from 70% to 0% B, 0.81−1.1 min reconditioning with 70% B. The flow rate was 4 mL/min, and continuous sampling of the first-dimension effluent was made by two 20 μL loops (mounted on the 10-port valve). UV−vis detection at 315 nm

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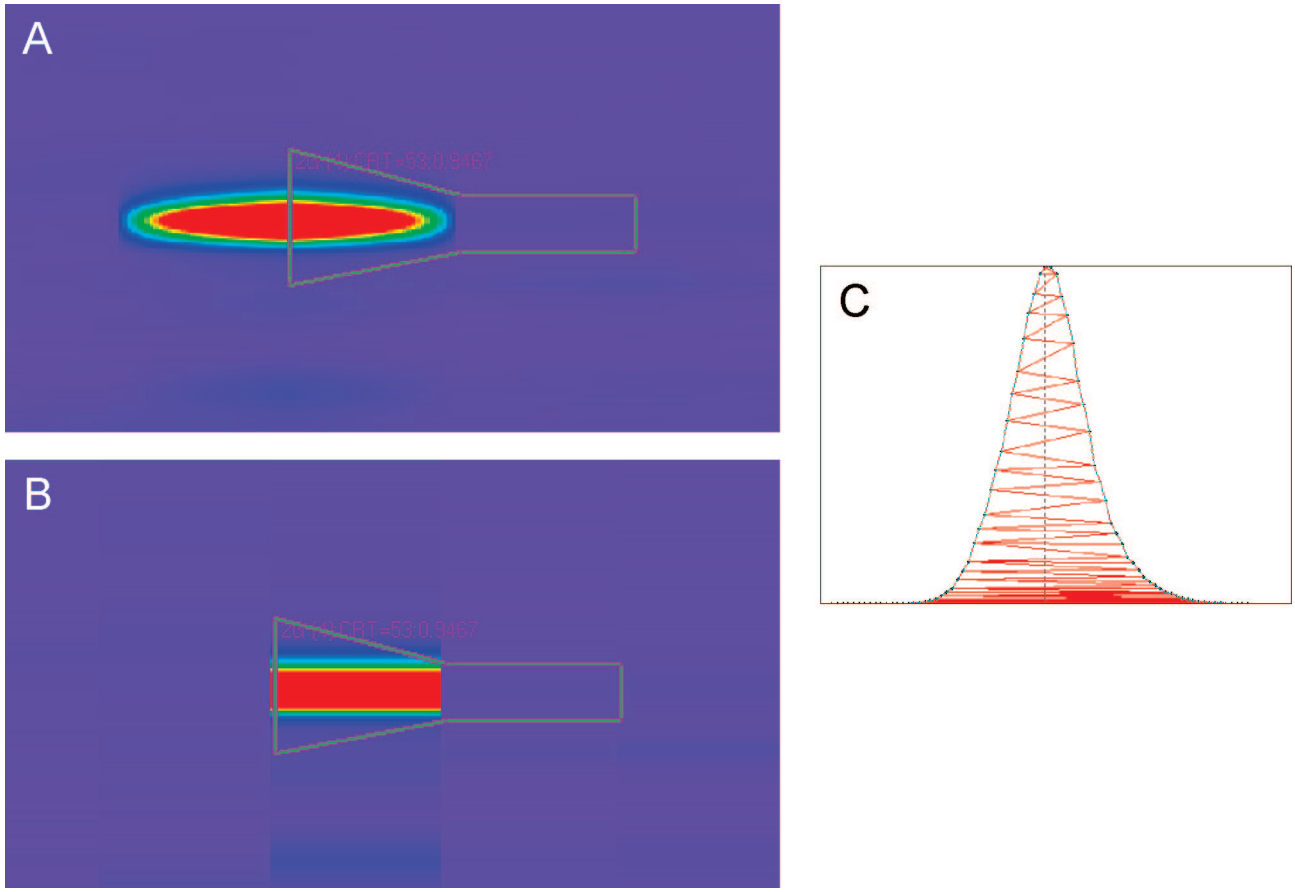

Figure 1. Integration area of a given peak when it is shown with (A) or without graphical interpolation (B), and example of the integration process for a second-dimension peak (C).

(sampling rate 25 Hz) was used. The modulation time was 66 s (1.1 min), and the temperature of the column oven was set at 30 °C. The back-pressure values reached were the following: first dimension, 10 bar (first-dimension column plus loop); second dimension, 110 bar (maximum value, loop plus second-dimension column).

LC×LC Analyses of Standard Mixture. In this case, a mixture of *n*-hexane and ethyl alcohol (98:2) was used as mobile phase in the first dimension. The flow rate was 15 μL/min, and the 2 μL injections were made by the autosampler. In the second dimension, the mobile phases consisted of acetonitrile (A) and water (B), following a 1.1 min gradient: 0.1 min 70% B, 0.15 min 50% B, 0.3 min 30% B, 0.5 min 0% B, 0.9 min 0% B, 0.91−1.1 min reconditioning with 70% B. The flow rate was 4 mL/min, and continuous sampling of the first-dimension effluent was made by two 17 μL loops (mounted on the 10-port valve). A DAD operated at a 12.5 Hz sampling rate was used in the 190−300 nm range, and the chromatograms were extracted at 212 nm. The modulation time was 66 s (1.1 min), and the temperature of the column oven was 30 °C. In this case, the back-pressure values reached were the following: first dimension, 9 bar (first-dimension column plus loop); second dimension, 110 bar (maximum value, loop plus second-dimension column).

RESULTS AND DISCUSSION

Software Development. The Chrom^{square} in-laboratory constructed software enabled both the conversion of the instrumental data into two- and three-dimensional chromatograms and direct peak integration. The latter procedure is based purely on chromatographic properties and differs from previously reported applications, where 2D peaks are integrated as images or considering peak volumes.[24–26] However, such integration methodologies may not produce correct results, because the programs employed represent bidimensional chromatograms as interpolated 2D plots, with the aim of showing three-dimensional images. As can be observed in Figure 1A, the shape of an interpolated peak can be far from real, greatly differing from a noninterpolated one (Figure 1B). Considering that the integration area defined in Figure 1 is derived from sequential second-dimension chromatograms, it can be concluded that an image-based integration, in such a situation, could be misleading. The results could be even worse when peaks eluting from the first-dimension separation are undersampled, that is, peaks that are sampled only once into the second dimension. Under these conditions, that are often employed, the error obtained using the image-based integration could be even greater. Also, in other comprehensive chromatographic techniques, several problems have arisen related to the image-based quantification.[26]

One of the greatest difficulties encountered if a conventional integration procedure is done relies on the proper determination

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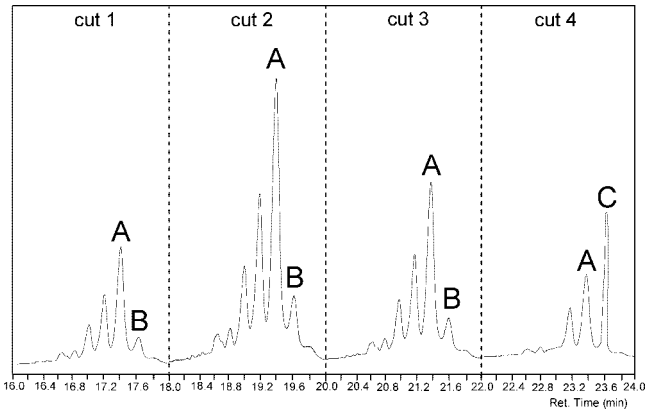

Figure 2. Four untransformed second-dimension chromatograms relative to an LC×LC application. The peaks marked as A, B, and C represent different compounds.

of peaks that belong to the same compound. In this respect, the software contained a novel algorithm that enabled the simultaneous identification and integration of analyte bands relative to the same solute. This fundamental data-handling process was achieved by considering detection data points during each modulation period. Obviously, both the modulation frequency and the detector sampling rate were involved. Hence, second-dimension peaks which eluted in the same data-point window (a correction factor can be applied), in consecutive modulation periods, were correctly identified by the program. Moreover, the reconstructed logical peak shape of peak groups produced by the same compound, in LC×LC experiments, was accounted for: for example, a compound eluting in the same second-dimension data-point window as a previous one, without following the expected logical peak pattern, would be considered as a different compound. This means that the second-dimension peaks corresponding to a single compound grouped together should have only a maximum. The effectiveness of this software feature is illustrated in Figure 2: in the 2 min second-dimension chromatograms four peaks are identified as A; on the contrary, although peak C eluted in the same data-point window as the three previous peaks identified as B, it was identified by the program as a different compound. In fact, peak C did not follow the logical peak profile as would have been expected considering the previous "cuts", as it would imply the existence of a second maximum. Such cases are often met in LC×LC applications.

The next step, once each second-dimension peak is assigned to a single compound, is the quantification process: all peak areas relative to each specific component group are measured and subjected to summation; thus, the total area for every 2D peak present in the 2D plot is derived. Areas are measured as follows: the program divides the area under each peak into what can be defined as "data-point triangles". The peak-apex data point is joined to the right-hand data point located immediately below; the latter point is connected to the first left-hand data point located immediately under the apex point; this left-hand point is linked to the second right-hand data point under the apex point, and so on, until all second-dimension peak data points have been connected (Figure 1C). The triangle areas are then determined and then summed. It is obvious that higher sampling frequencies will generate more triangles and, hence, greater accuracy. This integration mechanism was adequately validated. The results concerning this validation procedure are commented below. Besides this automatic integration function, the operator can select specific target components for quantitation. Furthermore, minimum and maximum peak area limits can be defined.

An additional function, defined as drift tolerance (expressed as a percentage of modulation time), enables the summation of peak areas with slight second-dimension retention time differences. Such experimental variations are quite normal in LC×LC analysis. For example, the four second-dimension retention times relative to peak A in Figure 2 are 17.395 (cut 1), 19.394, 21.396, and 23.412 (cut 4) min. Considering the 2 min modulation period, the retention time of peak A in cut 4 is slightly higher than that observed in the previous cuts. Nevertheless, the peak A area in cut 4 was correctly integrated and summed with the previous areas by setting a specific drift tolerance value of 2%.

The most important software functions have been described in this section. However, program development is ongoing in order to improve existing features and to add new ones.

LC×LC Analyses of Aurapten. The effectiveness of the software was evaluated in the LC×LC quantitative analysis of aurapten in grapefruit essential oil. The NPLC×RPLC analysis of citrus essential oils has been carried out by our group in previous studies.[16] Calibration curves were determined at six concentration levels (5−200 ppm) using coumarin (100 ppm) as internal standard and analyzing each solution three times consecutively. The aurapten concentration was plotted against both the aurapten peak area and the aurapten/coumarin (A/C) peak area ratio. The linearity attained was satisfactory with correlation coefficient values (R^2) of 0.9987 and 0.9995 obtained in the former and latter cases, respectively. A calibration curve was constructed in conventional LC applications through the same procedure; a correlation coefficient of 0.9991 was attained (also using the A/C peak area ratio).

Limits of detection (LOD) and quantitation (LOQ) were determined by using the untransformed LC×LC chromatogram, at signal-to-noise ratio (S/N) levels of 3 and 6, respectively. Although bidimensional chromatograms are very useful for a visual evaluation of the outcome of an LC×LC application, the raw chromatogram must be used for LOD−LOQ measurements. A series of issues must be considered when calculating such validation values in LC×LC experiments; in conventional LC the procedure is straightforward as a single peak height is related to baseline noise. In LC×LC analysis the same thing can occur, viz., a low-amount compound can be detected in a single slice (see peak I in Figure 3). A further possibility is that a trace-amount component is modulated twice, and hence, it is possible to detect it in two consecutive second-dimension chromatograms (see peak II in Figure 3). These factors are obviously related to the experimental conditions but can also depend on intra-analysis retention time variations. For this reason, it would be necessary to take into account theoretically each peak as a possible single peak in the second-dimension chromatogram in order to compare precisely the LOD and LOQ values either to conventional LC or to other LC×LC separations. In the present research, the aurapten LOD and LOQ values, which were 0.10 and 0.21 ppm, respectively, were measured on the basis of a single-cut transfer to the second dimension. The conventional LC LOD and LOQ values were 0.05 and 0.10 ppm, respectively. The LC×LC loss in sensitivity,

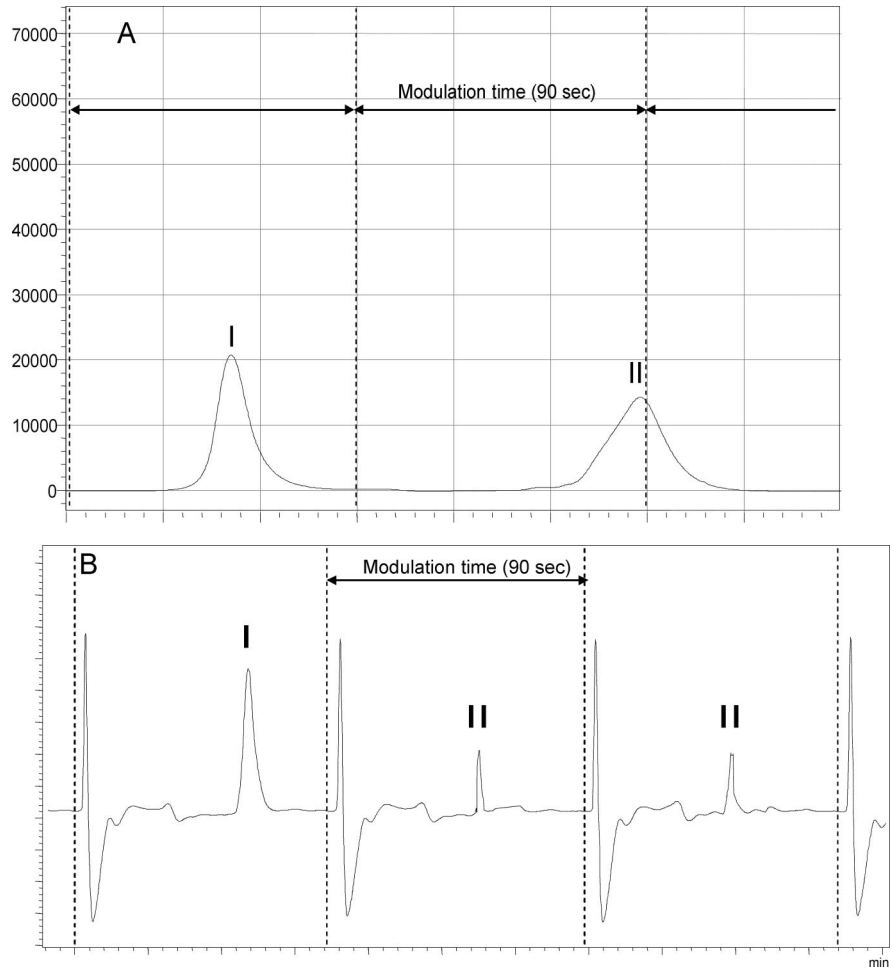

Figure 3. (A) Monodimensional chromatogram for compounds I and II and (B) single modulation for peak I and double modulation for peak II.

Table 1. Precision and Accuracy of the NPLC×RPLC Method for Quantitative Determination of Aurapten in Grapefruit Essential Oil

	concn of aurapten (ppm)		SD	error
	added	determined[a]	(ppm)	(%)
solution of standards I	70.0	68.8 ± 0.28[b]	0.23	1.7
solution of standards II	75.0	73.2 ± 1.6	1.03	2.4
orange essential oil I	50.0	51.2 ± 1.3	0.51	2.5
orange essential oil II	100.0	97.3 ± 0.9	0.34	2.7

[a] Calculated by using the equation $y = 0.0148x - 0.0168$ of the A/C calibration curve. [b] Average value ± standard deviation (95% confidence level).

approximately 2 times lower than in the single-column experiment, is due to sample dilution during the secondary separation. Correction factors were also derived and showed a good agreement between the monodimensional (0.76) and multidimensional (0.71) methods.

LC×LC peak area precision (n = 3) was also evaluated considering the A/C ratio at all six concentration levels: RSD% values ranged from 0.1% to 3.0%. When all 18 calibration curve analyses are considered, RSD% of the area of coumarin was 5.0%. Although the error/difference of the first RSD values includes mainly the instrumental error, the second one includes also the error of the human factor and therefore is slightly bigger.

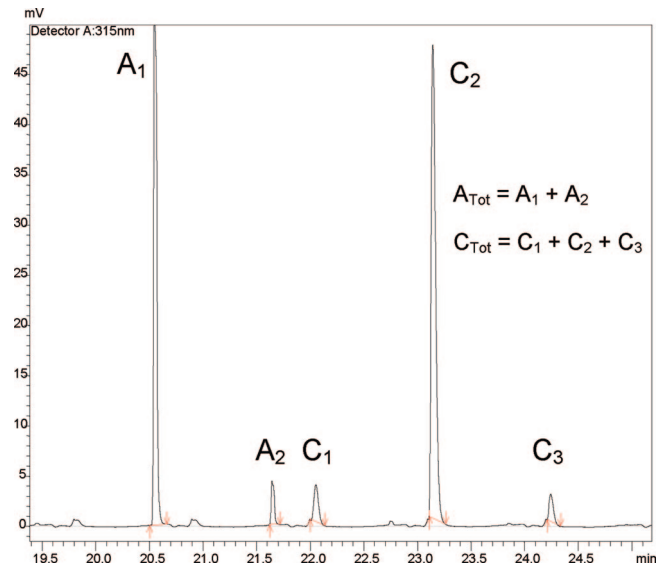

Figure 4. Raw chromatogram of an orange essential oil LC×LC analysis. The areas shown were determined and summed up manually to calculate the total areas of aurapten (A) and coumarin (C).

During the next phase, method accuracy was measured with and without matrix effects; in the latter case solutions spiked with a known amount of aurapten were prepared, whereas in the former

Table 2. Concentration Ranges, Equations, and Correlation Coefficients Relative to the Calibration Curves Derived for the Compounds Contained in the Standard Mixture

compd	concn range (ppm)[a]	equation[b]	R^2 [c]
toluene	4.9−494.0	$y = 26298x + 296844$	0.9987
hexylbenzene	5.0−499.8	$y = 27958x + 226393$	0.9991
cinnamaldehyde	5.0−499.9	$y = 16742x + 88566$	0.9995
amylcinnamaldehyde	5.0−504.0	$y = 21396x + 256641$	0.9997
coumarin	4.9−493.5	$y = 47049x + 2127616$	0.9995
cinnamyl alcohol	5.0−495.3	$y = 35875x + 183347$	0.9993

[a] Seven different concentrations were used for each calibration curve (except six for hexylbenzene), and each concentration was injected in triplicate. [b] y, peak area; x, concentration given in ppm. [c] R^2, correlation coefficient.

case an orange essential oil, again spiked with known amounts of aurapten (aurapten is not contained in this citrus product), was subjected to analysis. The relative error values for the different samples, reported in Table 1, were always more than satisfactory.

To further demonstrate the ability and to validate the integration procedure of our software, both, calibration curves and aurapten quantification were recalculated measuring the areas of the second-dimension peaks individually using the instruments' software (LCSolution, Shimadzu) and summing up manually those belonging to a given peak, as can be observed in Figure 4. The results of the aurapten quantification procedure using the conventional LC software spiking with 50 and 100 ppm of aurapten were 51.2 and 97.5 ppm, respectively. These values were compared to those shown in Table 1 obtained with the LC×LC software. The Student's t test confirmed that both measurements were not statistically different (95% confidence interval), thus demonstrating that the integration mechanism devised in the new software produced accurate results.

Finally, a genuine grapefruit oil was spiked with the internal standard and analyzed for the quantitative determination of aurapten in LC×LC and conventional LC experiments. Obviously, this double application was carried out for comparative reasons as the quantification of a given compound by comprehensive LC×LC, that can easily be separated through conventional LC, is of no practical use. The aurapten concentration in grapefruit oil determined by the two methods differed minimally, namely, 9475 (conventional LC) and 9567 ppm (LC×LC). The Student's t test was used and confirmed the fact that, at a 95% confidence level, there was no statistical difference between the two values. Thus, the ability of the methodology presented including internal standard calibration and a dedicated software to quantify valuable compounds from natural matrixes by comprehensive LC×LC has been successfully demonstrated.

Quantitative Analysis of Hydrocarbons and Allergens. At this point, the software was employed in the simultaneous LC×LC quantification of a mixture of pure standard compounds: toluene, hexylbenzene, cinnamaldehyde, amylcinnamaldehyde, coumarin, and cinnamyl alcohol. Seven-point calibration curves (except for hexylbenzene, where six concentration levels were used) were constructed ($n = 3$), with correlation coefficients never lower than 0.9987 (Table 2).

The method accuracy was again verified in an LC×LC analysis on all six standard components; the bidimensional chromatogram relative to this application is illustrated in Figure 5. The measured relative error was satisfactory (less than 4%) for all compounds except for hexylbenzene and amylcinnamaldehyde, where 15%-plus values were attained. Probably, this negative issue was due to the baseline drift caused by the mobile phase employed, as can be observed in Figure 5 (see the upper blue band).

In order to assess the influence of the baseline drift on quantitation, the previous standard mixture was reduced to three compounds on the basis of their 2D location: coumarin (52.50 ppm)—located far from the drift, toluene (61.75 ppm)—situated near the drift, and hexylbenzene (51.00 ppm)—located in the middle of the drift. A 51.47 ppm concentration was found for coumarin, one of 65.34 ppm for toluene, and 63.54 ppm for hexylbenzene (relative error 24.6%). As is evident, the problems encountered in the quantification of hexylbenzene persisted. With the aim of improving this disappointing outcome, a blank analysis

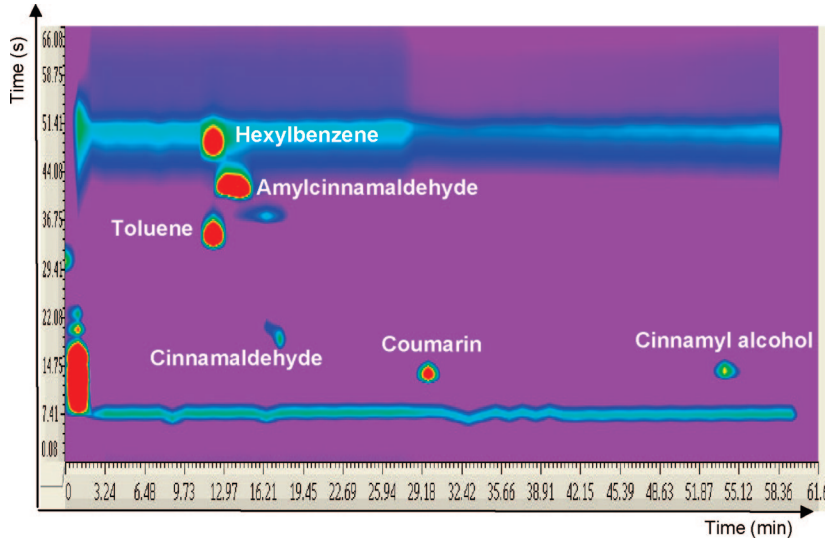

Figure 5. Two-dimensional plot relative to the LC×LC analysis performed using the standard mixture composed by toluene (61.7 ppm), cinnamaldehyde (64.8 ppm), amylcinnamaldehyde (126.0 ppm), hexylbenzene (51.0 ppm), coumarin (26.3 ppm), and cinnamyl alcohol (31.8 ppm) diluted in a mixture of *n*-hexane/ethanol (98:2). For experimental conditions see text.

Table 3. Concentration Ranges, Equations, and Correlation Coefficients Relative to the Additional Calibration Curves Derived for Three Compounds Contained in the Standard Mixture[a]

compd	concn range (ppm)[b]	equation[c]	R^2 [d]	real concn (ppm)	exptl concn (ppm)[e]	error (%)
toluene	4.9−494.0	$y = 25040x + 48013$	0.9998	61.8	59.0 ± 0.59	4.5
hexylbenzene	5.0−499.8	$y = 17881x + 32894$	0.9997	51.0	51.5 ± 2.75	1.0
coumarin	4.9−493.5	$y = 46776x + 69126$	0.9993	52.5	52.2 ± 1.11	0.6

[a] Method accuracy results derived by using the additional calibration curves. [b] Seven different concentrations were used for each calibration curve (except six for hexylbenzene), and each concentration was injected in triplicate. [c] y, peak area; x, concentration given in ppm. [d] R^2, correlation coefficient. [e] Average value ± standard deviation (95% confidence level).

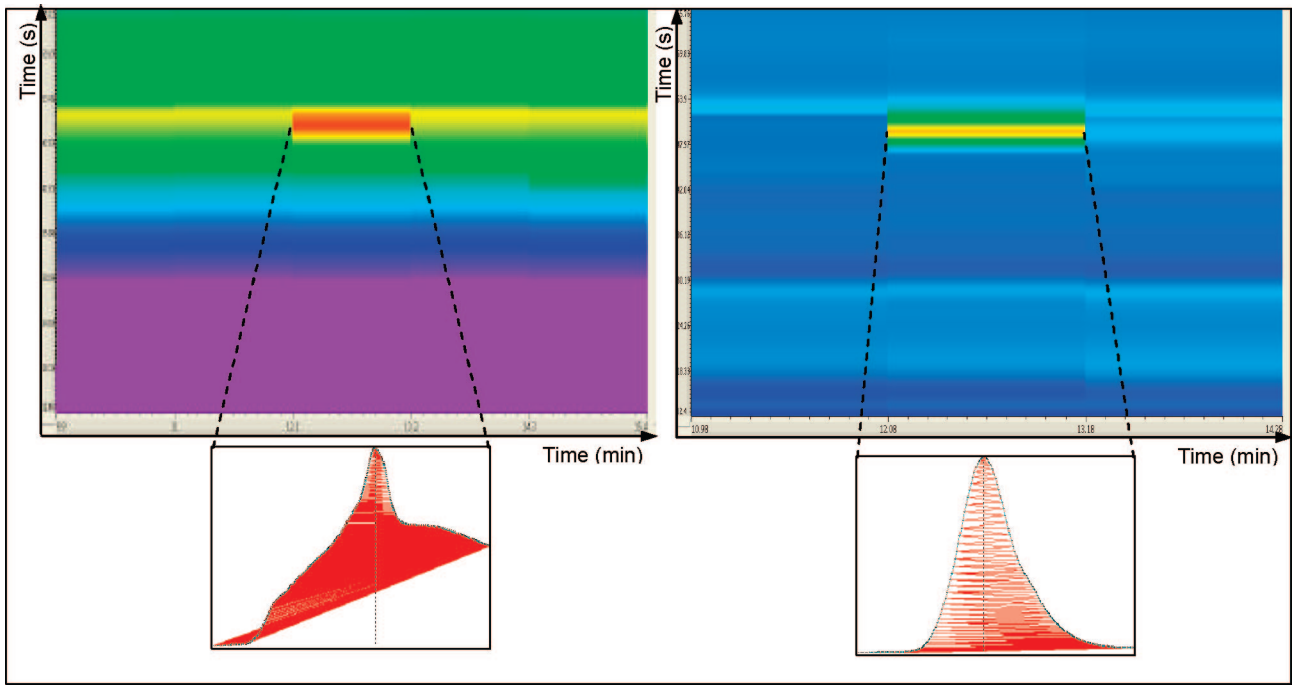

Figure 6. Two 2D chromatogram expansions relative to the hexylbenzene peak prior to and after the correction process. Also shown are the untransformed and integrated second-dimension chromatogram hexylbenzene peaks.

was performed prior to the construction of new calibration curves, carried out this time by using the baseline subtraction function of the software. Satisfactory correlation coefficients were attained, with values never lower than 0.9993 (Table 3). At this point, method accuracy was determined a second time; this time the relative error values were very good in all cases, in particular for hexylbenzene which reached a value of less than 1%.

The effect of baseline subtraction can be appreciated in Figure 6, which reports two 2D chromatogram expansions relative to the hexylbenzene peak prior to and after this correction process. Also illustrated are the untransformed and integrated second-dimension chromatogram hexylbenzene peaks. It is evident from the figure that (a) correct peak integration is problematic when a peaks elutes at the same time as a rapidly raising baseline; (b) LC×LC baseline subtraction can be very useful for proper quantification. Baseline drift had also a great influence on sensitivity: prior to baseline correction the LOD and LOQ values were 9.79 and 19.59 ppm, respectively; after baseline subtraction these values decreased almost 50 times, reaching 0.20 and 0.40 ppm, respectively.

CONCLUSIONS

In the present work, different aspects related to quantification procedures in comprehensive LC×LC by using a dedicated software have been discussed for the first time. The suitability of comprehensive LC×LC for the quantitative determination of several compounds using both the external standard and the internal standard method has been shown. Moreover, the usefulness of baseline subtraction in LC×LC applications has been demonstrated. Future research will be devoted to software improvement−development and to its use in complex real-world sample LC×LC experiments.

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