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Patterns in the precision of quantitative data from multicomponent gas chromatographic or gas chromatographic–mass spectrometric analysis☆

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Abstract

The dispersion of the quantitative results in the analysis of volatile compounds from multicomponent mixtures by different fractionation techniques (solid-phase microextraction and direct thermal desorption) followed by GC or GC–MS presents nonrandom patterns related to the existence of different factors in the fractionation process or in the chromatographic separation which affect, to a different extent, the recovery of the sample components. Statistical techniques have been used to show the relative importance of these factors. The improvement in data precision achieved by using volatile compound concentration ratios is discussed.
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Keywords: Principal component analysis; Precision; Statistical analysis

1. Introduction

Volatile compounds are usually present in natural products as complex mixtures of components with different functional groups. Although generally in low concentrations, they are responsible for many sample properties such as plant and food aroma. Hence the interest in the qualitative and quantitative determination of such compounds.

Analysis of volatile mixtures is usually carried out by gas chromatography (GC) or GC–mass spectrometry (GC–MS). GC has a high resolving power and is especially suited to the analysis of volatile compounds. MS affords additional qualitative information, necessary in many cases for identification. Quantitative analysis is based on peak area measures (usually obtained from flame ionization detection (FID) in GC analysis and from total ion current (TIC) or selective-ion monitoring in GC–MS). In both cases a calibration procedure using a suitable standard is required for an accurate determination.

For GC or GC–MS analysis of a sample, non-volatile components which could remain in the injection system causing decomposition artefacts must first be removed. This first step in the analysis of volatile compounds is usually a fractionation process in which volatiles isolated from the non-volatile matrix are injected into the GC column by either an on-line or an off-line procedure.

Solid-phase microextraction (SPME) and thermal

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desorption are widely used techniques for the fractionation of volatile compounds. In SPME, volatiles are submitted to a partition and/or adsorption process between the sample and a polymeric coating on a fused-silica fiber [1]. The fiber is then inserted in the GC injector, where volatile compounds are desorbed at high temperature to enter the chromatographic column. SPME offers the advantages of low cost, simplicity of operation and versatility.

In direct thermal desorption (DTD), volatiles are isolated from the solid sample by sweeping with an inert gas at a controlled temperature and then injected into the gas chromatographic column [2]. DTD is a fast technique which requires only a very small amount of sample and allows automatic operation, but it cannot be applied to samples which contain water or thermally unstable compounds [3].

Although the final analytical step in the techniques above mentioned is usually GC or GC–MS, results can differ considerably, since component recovery depends not only on the sampling procedure, the technique used and the operational conditions, but also on the volatility, polarity and other physical and chemical characteristics of the compounds.

Calibration based on the analysis of standard compounds is not easy in quantitative determinations when the samples analysed contain a high number of volatile components and many of them are not available. For this reason, when quantitative data are used for comparative or characterization purposes, the results are often obtained as relative concentration values, and precision rather than accuracy is considered the most important parameter. Standard deviation, or more frequently relative standard deviation (RSD), are usually employed as estimators of the dispersion for quantitative values and to express the precision of a series of measures [4–7].

Methods based on automatic procedures, such as DTD, usually afford precise results, but the main handicap of SPME is probably lack of precision when using a manual injection system. This lack of precision affects compounds with different characteristics in different ways.

Individual or overall precision are parameters frequently used to evaluate or compare the quality of an analytical method. Precision is also considered to be one of the most important variables requiring optimization in the selection of operational conditions, since a low precision is assumed to be the result of inadequate control of experimental parameters. Little attention has been paid to the relations among the different dispersions presented by different compounds in a complex mixture, as they are generally supposed to be independent and randomly distributed. However, principal component analysis (PCA) has been found to be a useful tool for distinguishing between different operation conditions and among the analytical responses of different compounds in SPME analysis [8].

The objective of this study was to evaluate the application of statistical analysis to the study of precision in GC or GC–MS analysis of multicomponent mixtures with a view to identifying dispersion patterns that cannot be attributed to random causes.

2. Experimental

2.1. Samples

An Italian chestnut honey sample was selected for volatile analysis in the SPME study. Commercial cumin seeds (Santiveri, Spain) were left to dry, homogenized in a blender and then directly analysed by DTD–GC–MS.

2.2. Solid-phase microextraction followed by GC

SPME headspace sampling was done on a manual SPME holder equipped with an 85-μm polyacrylate fiber (both from Supelco, Bellefonte, PA, USA). Fiber was conditioned at 300 °C before analysis until no interfering peaks were obtained in a blank run.

The experimental procedure was as follows: 3 g of honey was exactly weighted into a 10-ml vial and carefully homogenized with 0.5 g of anhydrous sodium sulphate (previously conditioned overnight at 80 °C). The vial was immediately sealed by means of a septum and a metallic ring.

After an equilibrium time of 30 min at 70 °C (with hand shaking after 10 and 20 min), fiber was exposed in the headspace of honey sample for 20 min.

GC analyses ($n=8$ replicates) were performed on a gas chromatograph (Perkin-Elmer AutoSystem, Norwalk, CT, USA) equipped with a FID. The SPME fiber was desorbed (18 mm deep on the SPME scale)

at 250 °C for 3 min in splitless mode using a 0.75 mm narrow bore liner (1:50 split ratio). Chromatographic separation was carried out on a 30 m×0.32 mm I.D., 0.5 μm film thickness HP-Innowax capillary column (Agilent Technologies, USA). The oven was temperature programmed from 50 °C (4 min) to 230 °C (10 min) at 10 °C min^{-1} and up to 250 °C (5 min) at 10 °C min^{-1}. He at ~1 ml min^{-1} was used as carrier gas. The FID temperature was 250 °C.

CHROMCARD for Windows version 1.20 (CE Instruments, Milan, Italy) was used for data acquisition and processing.

2.3. DTD–GC–MS

Volatile fractionation was carried out in an ATD 400 automatic thermal desorption unit (Perkin-Elmer). A homogenized cumin sample (10–20 mg) was put into a PTFE tube (52×4 mm I.D.×4.5 mm O.D.) which was then placed into a stainless steel desorption cartridge (89 mm×4.5 mm I.D.×6.5 mm O.D.). Volatile compounds were desorbed under a 40 ml min^{-1} helium flow at 180 °C for 15 min and then cryofocused on a Tenax GC 60–80 mesh trap at −30 °C. This trap was heated up to 320 °C at ~40 °C s^{-1} and kept at the maximum temperature for 4 min. The desorbed volatiles were transferred to the GC column through a fused-silica line heated at 225 °C. Inlet and outlet split flows were 100 ml min^{-1}. Other conditions are detailed elsewhere [9].

The ATD 400 was connected on line to a GC 8000 gas chromatograph (Fisons, Milan, Italy) coupled to an MD 800 quadrupole mass detector (Fisons, Manchester, UK). A methyl silicone SPB-1 capillary column (27 m×0.25 mm I.D., 0.25 μm film thickness) was temperature programmed from 60 to 180 °C (at 3 °C min^{-1}) and then to 250 °C (at 8 °C min^{-1}) for 5 min. Helium at ~1 ml min^{-1} was used as carrier gas. Blanks were run after each of the ten replications.

Mass spectra were recorded in the electron impact (EI) mode at 70 eV, scanning the 38–350 m/z range. Interface and source temperature were 280 and 230 °C, respectively.

Data acquisition and data processing were carried out using the MASSLAB software, version 1.4 (Finnigan, Manchester, UK).

2.4. Characterization and quantitative determination

Characterization was based on retention times (when using FID) or on retention times and mass spectral data (when using GC–MS). GC–MS identifications were carried out by comparison of experimental mass spectra with those of the Wiley library [10] and using standard compounds when available.

Relative quantitative values (percentage of total volatile composition) were calculated from the peak areas of FID or TIC profiles. Differences in response factor were not taken into account.

2.5. Statistical data analysis

2.5.1. Experimental data

Percent quantitative results were presented as two data matrices of 69 peaks×8 replicates (FID peak areas from SPME and GC analysis) and 41 peaks×10 replicates (TIC peak areas from DTD–GC–MS analysis).

Mean and RSD values were calculated for each of the peaks selected for quantitation. Residual matrices were obtained by subtracting mean values from the original values for each run.

2.5.2. Simulated data

Simulated data ($n=10$ simulations) were obtained using a VISUALBASIC program developed at our laboratory and based on the Rnd function [12]. They presented a normal distribution, with the same mean and RSD values as those obtained from experimental data. Residual data matrices from SPME (or DTD) simulated data were obtained as previously described for experimental data.

2.5.3. Volatile component ratios

Ratios between percent concentrations were calculated for all possible pairs of volatile components quantified in each replicate. RSD values were calculated for these ratios.

2.5.4. Data processing

Statistical analysis was carried out by using the BMDP statistical package for PC [11]: calculation of univariate parameters (program 2D), principal com-

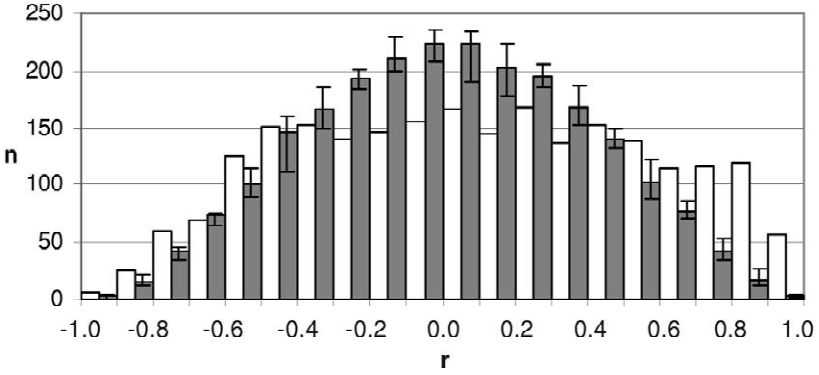

Fig. 1. Distribution of 2346 correlation coefficients (r) obtained for experimental (white bars) and simulated (grey bars) data matrices from SPME and GC analysis of chestnut honey volatiles.

ponent analysis and correlation coefficients (program 4M) and one-sample t tests for significance of differences (program 3D).

3. Results

3.1. Solid-phase microextraction

Volatiles in chestnut honey were analysed as described in Experimental. Sixty-nine peaks were characterized from their retention times, and quantitative data from the chromatographic FID profile were obtained for each peak in the eight replicates. Area values were normalized as percentage of total peak area. Experimental and simulated residual matrices were calculated from percent volatile data and submitted to PCA.

Fig. 1 plots the distribution of the 2346 correlation coefficients obtained for experimental (white bars) and simulated (grey bars) data in a given interval; maximum and minimum values for the ten sets of simulated values are also shown in this figure by error bars.

A bar plot is also presented in Fig. 2 for the eigenvalues of the first seven principal components obtained in the PCA analysis of experimental and simulated data. Bar height represents the variance explained by each principal component for experimental (white bars) and simulated (grey bars) data.

Loading values for the first three principal components are also summarized in the plot of Fig. 3.

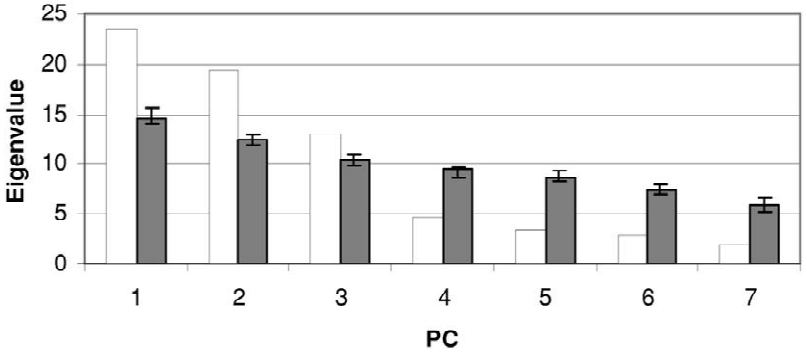

Fig. 2. Eigenvalues obtained for the first seven principal components (experimental data, white bars; simulated data, grey bars) in the PCA analysis of honey volatiles by SPME and GC.

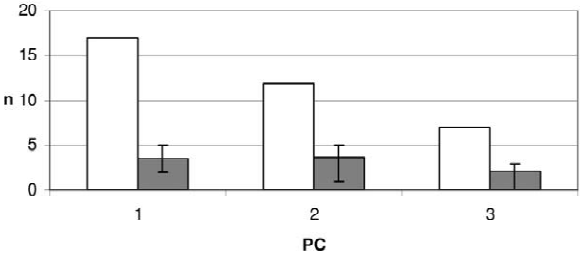

Fig. 3. Number of loadings with absolute value higher than 0.8 in the PCA analysis of chestnut honey volatiles by SPME and GC (experimental data, white bars; simulated data, grey bars).

Bars correspond to each principal component number, and their height is the number (*n*) of volatile compounds which present an absolute loading value higher than 0.8 for this principal component. As before, white bars are used for experimental values and grey bars for simulated data.

RSD values for the experimental data obtained from the sixty-nine compounds quantified in eight replicates ranged from 6.8 to 150%. The highest RSD values observed for some compounds were probably caused by artefacts from the SPME fiber or by chromatographic problems.

Ratios between the sixty-nine compound concentrations were calculated from the normalized data matrix, and RSD values were obtained for all these ratios. Twenty-eight experimental ratios presented an RSD value $<3\%$. When the same procedure was applied to the simulated data, only one from the ten simulations produced two ratios with RSD below 3%.

3.2. Direct thermal desorption

Volatile compounds were determined from a commercial cumin sample using direct thermal desorption coupled to GC–MS.

Forty-one compounds were characterized from their Kovats index and mass spectra, and most of them were identified. Quantitative results were calculated directly from TIC peak areas. Mean and RSD values for both experimental and simulated results were also calculated from the data matrices, and residual matrices were obtained as previously described.

Program 4M of the BMDP package afforded 820 correlation coefficients between all the possible compound pairs. The plot in Fig. 4 displays the distribution of their experimental values (white bars) and that of the coefficients obtained from ten simulated data matrices (grey bars).

Fig. 5 presents a bar plot with the eigenvalues of the first nine principal components, calculated by applying PCA to the experimental and simulated data matrices. The histogram in Fig. 6 shows the distribution of the highest loadings for the first three principal components: bar height indicates the number (*n*) of compounds in which the absolute loading value is higher than 0.7. As before, white bars correspond to experimental and grey bars to simulated data.

The experimental RSD values ranged from 3.5 to 67.8% for 41 compounds quantitatively characterized in ten replicate analysis.

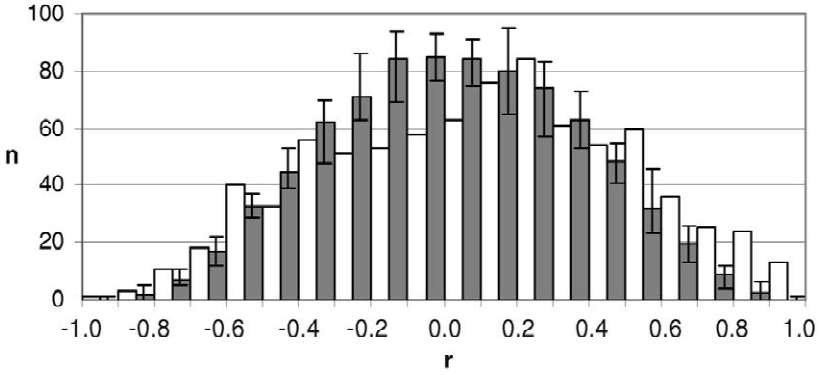

Fig. 4. Distribution of 820 correlation coefficients (*r*) obtained for experimental (white bars) and simulated (grey bars) data matrices from DTD–GC–MS analysis of cumin volatiles.

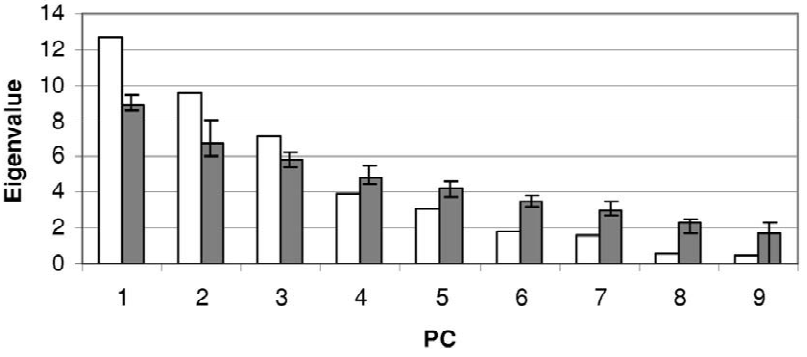

Fig. 5. Eigenvalues obtained for the first nine principal components (experimental data, white bars; simulated data, grey bars) in the PCA analysis of cumin volatiles by DTD–GC–MS.

RSD values were also calculated for all the ratios between pairs of compounds obtained; 30 of these values were <3% for experimental data. No values <3% were found in eight of the simulated data sets, while only two and one values appeared in the two remaining data sets.

4. Discussion

Analytical determinations present quantitative errors which can affect different compounds in different ways. Unless suitable standards showing a behaviour similar to that of the compounds being analysed are available, this discrimination decreases not only the accuracy but also the precision, since the magnitude of the errors can differ from one analysis to another.

In GC multicomponent analyses there are commonly groups of peaks whose profile for different injections is very similar, while important relative variations are observed for others. Fig. 7 plots four replications in the analysis of volatile compounds from a chestnut honey sample using SPME followed by GC. In a few cases (such as peaks marked c in Fig. 7), the origin is probably the analytical system (SPME fiber, septum or column bleed), as their intensity is independent of sample considerations and they also appear in blank runs. In most cases, however, groups of peaks present a consistent profile (peaks marked a) which differs from the profiles of peaks in other groups (e.g. peaks marked b). If the relative recovery for these groups depends both on compound characteristics and on operational conditions, incomplete control of the latter will result in variable discrimination and hence in loss of precision.

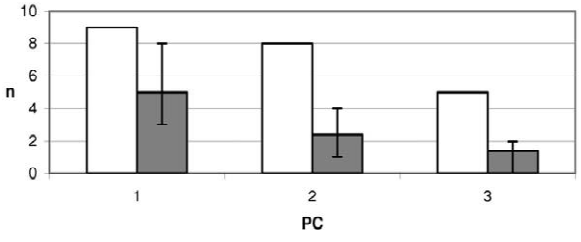

Fig. 6. Number of loadings with absolute value higher than 0.8 in the PCA analysis of cumin volatiles by DTD–GC–MS (experimental data, white bars; simulated data, grey bars).

Chemical and physical characteristics (polarity, molecular mass, volatility) of the compounds in a multicomponent mixture, generally considered to be responsible for discrimination, can change continuously. If the effects of each factor were isolated, they could be studied; however, this is not possible since their effect on precision may be cumulative.

The approach followed in this study was to compare several statistical parameters calculated from experimental data with those obtained by random simulations which present a similar overall dispersion.

4.1. Correlation coefficients

The correlation coefficient (r) is the simplest measure of the relationship between two sets of

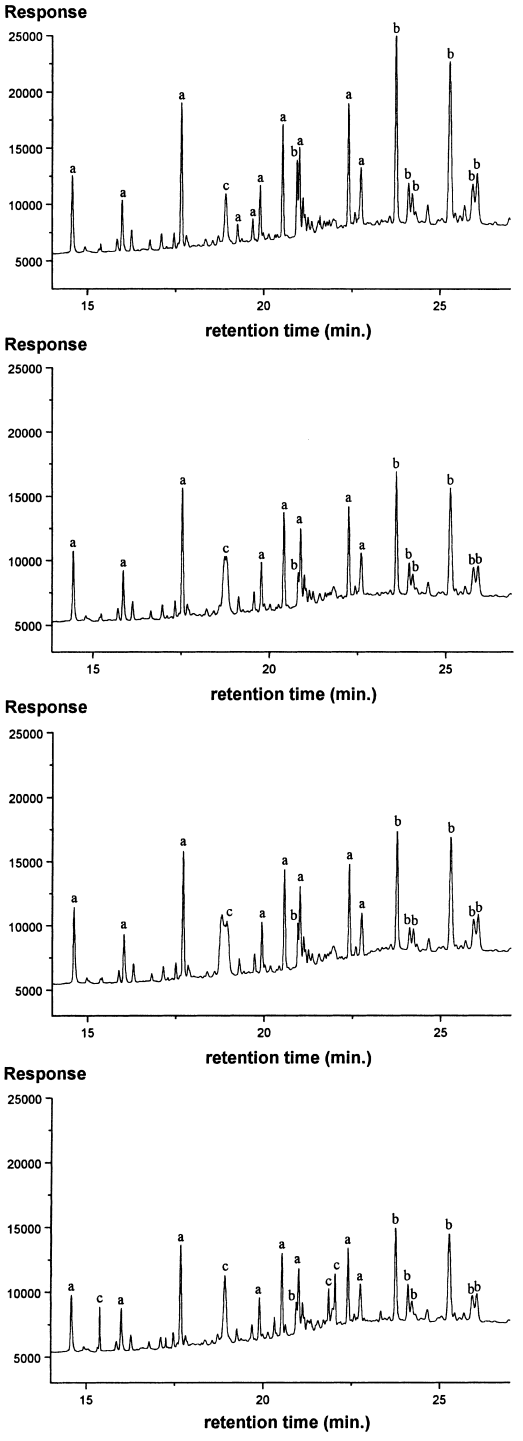

Fig. 7. GC profiles ($n=4$ replicates) for the volatile fractions obtained by SPME from a chestnut honey sample. Peaks marked a, b and c are discussed in the text.

values. In a simulated matrix, where residuals are supposed to be independent, correlation coefficients should present a low absolute value. However, since $n \cdot (n-1)/2$ values of r are obtained for n compounds, several high positive and negative values are expected for high values of n. This type of distribution appears in the plots for simulated data in Figs. 1 (honey, SPME) and 4 (cumin, DTD).

On the other hand, the distribution of r values obtained from experimental data presents distinctive features, which are clearly shown in the plots of Figs. 1 and 4. The number of r values with low absolute value (centre of the plot) is lower for experimental data, negative values in the -1 to -0.5 range are slightly more frequent in experimental data, and high positive values (in the 0.6–1 range) are clearly more frequent in experimental data for both SPME (honey, Fig. 1) and DTD (cumin, Fig. 4) data.

Differences between experimental and simulated correlation coefficient frequencies are highly significant for positive values. For SPME data, t values calculated using the one-sample t test for the differences in frequencies in the 0.7–1.0 range, were between 31.6 and 137.2 (probability of being produced by random causes $P<0.01\%$). For DTD data, t values are between 18.4 and 96.0, also with $P<0.01\%$).

In order to interpret these results, we need to identify groups of compounds that share a particular behaviour. A useful tool for this purpose is principal component analysis (PCA).

4.2. Principal component analysis

With factor analysis, the variance of a data matrix can be assigned to several mathematical factors, each of which is a linear combination of the original variables. In the PCA technique factors are calculated to explain the maximum proportion of data variance. The eigenvalues obtained are a measure of the amount of variance explained by each factor.

The variance in an experimental data matrix is caused by a combination of random and nonrandom ('systematic') factors. They cannot be estimated accurately without prior knowledge of their characteristics, but PCA affords information on their relative importance and on the associated variables

(compounds) for each factor, taking into account that several experimental factors can overlap in each statistical component.

Fig. 2 plots the eigenvalues calculated using PCA from the experimental (white bars) and simulated (grey bars) results for the SPME fractionation of chestnut honey volatiles. The most important feature of Fig. 2 is the gap between the eigenvalues of third and fourth components of experimental data, which do not appear in the PCA of simulated data. Eigenvalues of components 4–7 decrease in a similar way for both data sets.

The one-sample *t* test provides for the differences between the three first eigenvalues of experimental and simulated data *t* values between 16.7 and 57.3 ($P < 0.01\%$, highly significant).

Simulated data eigenvalues show a continuous decrease from principal component 1 to component 7. A similar tendency is observed in experimental data for the lower order (4–7) eigenvalues, that are supposed to correspond to dispersion caused by random factors or for singular variables.

Eigenvalues 4–7 from experimental data are about a 40% of those from simulated data. If we suppose that this percentage is valid for all the eigenvalue range, we can estimate that nonrandom variance is about 75% of eigenvalues 1 and 2, and about 70% of eigenvalue 3.

An analogous procedure was applied for comparison of the eigenvalues obtained in the PCA analysis of experimental and simulated DTD data matrices. Differences between eigenvalues are also significant for the three first components: *t* values range between 14.0 and 48.4 ($P < 0.01\%$). Fig. 5 shows, in a similar format to that of Fig. 2, the tendency observed for experimental and simulated eigenvalues for the nine first principal components calculated.

In this case, the amount of variance associated to random factors in experimental data for the principal components 8–9 is about 30% of that of simulated data sets. The estimated proportion of nonrandom variance results to be 80% of eigenvalues 1–2 and 75% of eigenvalue 3.

A study on the experimental factors possibly associated with the most important eigenvalues requires to know how the different volatile compounds contribute to them. Loadings are the correlations of the principal components with the original variables: an experimental factor that positively affects the response of a group of volatile compounds will produce positive loadings for them in the corresponding principal component. For a given compound, the value of the loading depends on the intensity of the effect, taking into account that a single principal component can be affected by several experimental factors.

As explained in the discussion of correlation coefficients, the large number of data obtained in multicomponent analysis can produce random correlations and, in turn, apparently significant loadings. For this reason, a comparison with simulated data is necessary to estimate the real contribution of nonrandom factors to loading values.

Fig. 3 is a bar plot comparing the number of loadings higher than 0.8 in the first, second and third principal components for experimental (white bars) and simulated (grey bars) SPME data. The number of high loadings in the three first principal components is consistently greater for experimental data. The one-sample *t* test provides for the differences between experimental and simulated data *P* values $< 0.01\%$, proving that these differences are highly significant.

Fig. 6 indicates that an effect similar to that described in Fig. 3 exists for the loadings higher than 0.8 of the three first principal components calculated from DTD–GC–MS volatile data.

Correlation coefficients between compounds with high loadings present usually a high value. For instance, the DTD–GC–MS correlation coefficients for the twenty-one possible pairs among the seven peaks with loadings > 0.9 in the first principal component are always in the 0.7–0.9 range.

Compounds which present similar high loadings in the first principal components are presumably influenced in a similar way by one or more experimental factors. Both loading values and eigenvalues depend on the importance and extent of these factors.

Most of the compounds with loadings higher than 0.900 from DTD–GC–MS data were identified as sesquiterpenes (β-farnesene, germacrene, *trans*-caryophyllene, etc.) and eluted closely in the chromatographic column, showing that compounds with related physical or chemical properties present a similar dispersion pattern.

In the SPME–GC analysis of honey volatiles, identification from the retention time was not possible for most peaks. However, a study of the relationship between loading values and retention times shows that most peaks with a high loading for the second component eluted in the last part of the chromatogram, indicating a relatively low volatility.

4.3. Volatile component ratios

A sample can be characterized from its volatile component concentration using relative values. Percent concentrations relative to the total volatile amount are commonly used, but the ratios between the percent concentrations of two compounds are also useful. The parameters used for characterization purposes should be precise enough to discriminate between different samples.

The use of an internal standard affords absolute quantitative data, but if the advantages of relative values are to be maintained, matrix effects should be avoided and a compound with similar characteristics to those of the sample components analysed should be selected. When multicomponent samples are analysed, this last requirement is difficult unless several standards are included.

The use of ratios between relative compound concentrations in experimental data considerably improved their RSD values. RSD values for chestnut honey volatile concentrations were always $>7\%$, but when compound concentration ratios were used, 28 of them had RSD values $<3\%$. This increase in precision cannot statistically be caused by the high number of ratios, since the improvement of precision when using simulated data was far lower: only two ratios presented a RSD value $<3\%$ in one simulation.

Similar results were found for the DTD fractionation of cumin samples; while no RSD values $<3\%$ appeared in experimental or simulated data, when concentration ratios were used, up to 30 experimental values were below this limit.

The compound pairs which produce ratios with the best RSD values generally belong to compounds which present a high correlation coefficient between concentrations and which also present high loadings in the first principal components. All peaks used in the 28 ratios having an RSD$<3\%$ in the SPME data had PCA loadings >0.840 for the first principal component, and correlation coefficients between any pair of peaks in the set were >0.69 in every case. For DTD results, similar results were obtained: volatiles included in ratios with RSD values $<3\%$ had loadings >0.750 and correlation coefficients >0.72.

Table 1
Relative standard deviation (RSD) values for selected peaks in the analysis of chestnut honey volatiles by SPME and GC

RSD[a] (%)	Peak number (PC1)					Peak number (PC2)			
	7	17	22	27	38	54	56	57	69
A	12	11	10	10	11	17	9	21	20
B	5	3	2	2	–	25	27	15	27
C	15	16	15	14	15	11	–	13	12

[a] A, RSD calculated from percent data. $n=8$ replicates; B, RSD for concentration ratios using as reference peak 38. C, RSD for concentration ratios using as reference peak 56.

Table 1 lists the relative standard deviation for nine of the volatiles fractionated by SPME from a chestnut honey sample. The five peaks of the left group present high loadings for the first principal component, while the four peaks in the group at the right have high loadings for the second principal component. Row A corresponds to the original RSD values for these peaks. In row B, RSD values were calculated from the ratios between every peak and peak 38 (from the first group), while in row C peak 56 from the second group was selected as reference. In both cases there is a marked increase in precision when a peak belonging to the same group is selected as reference, while precision decreases when the reference peak belongs to another group. Selection of reduced groups of compounds from SPME simulated data matrices shows no significant improvement in data precision. Analogous results were obtained for DTD–GC–MS data.

5. Conclusions

Some experimental systematic factors that produce quantitative dispersion and hence loss of precision in the sampling, fractionation and gas chromatographic analysis of complex mixtures of volatile compounds can be studied from the patterns which emerge in the statistical processing of quantitative data. Eigen-

values obtained by PCA allow an estimation of the number of these factors and of the amount of dispersion that they produce; also, the relative importance of systematic and random factors can also be estimated for each principal component.

The values of the loadings can be used to identify chromatographic peaks influenced by systematic factors and to estimate the sign and intensity of this influence. An experimental basis for these factors could be based on the characterization or identification of these peaks and the study of their common physical or chemical characteristics.

Percent relative values are adequate for characterization purposes, where precision is the most important requirement. The use of suitable relative concentration ratios instead of relative percentage values increases this precision. However, when a higher precision is required for many sample compounds, different compounds must be selected as references in the concentration ratios, since improvement in precision is restricted to the peaks identified by PCA as having a dispersion behaviour similar to that of the reference.

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