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Direct continuous supercritical fluid extraction as a novel method of wine analysis
Comparison with conventional indirect extraction and implications for wine variety identification

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Abstract

Direct supercritical fluid extraction (SFE) of wines with carbon dioxide was compared to SFE of the sorbent used for solid-phase extraction of the same wine samples (SPE–SFE). Compared to SPE–SFE, the direct SFE results in a more specific and representative gas chromatographic fingerprint of the wine sample. The multivariate statistical processing of the direct SFE–GC data provides a clear-cut and sharp discrimination among the individual wine varieties while the discrimination based on the SPE–SFE–GC data is relatively poor. This finding reflects the adverse effects of additional analyte–sorbent interactions and sorption/desorption steps involved in SPE–SFE.
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1. Introduction

During the last 15 years, the emphasis in analytical applications of supercritical fluid extraction (SFE) has gradually shifted from the solid to the aqueous samples. One line of applications employed an indirect route consisting in solid-phase extraction (SPE) of the aqueous sample followed by SFE of the analytes from the sorbent [1]. Another line made use of direct SFE of the aqueous sample in a high-pressure vessel [2–5], and it was applied to diverse types of analytes including phenoxy acids [6], pesticides [7–9], herbicides [10], pharmaceuticals [11], steroids [12] or metal–ligand complexes [13–16]. The analyte recoveries obtained by the direct and indirect routes were compared to each other with various results [7,17] depending on experimental conditions and analyte type.

With a few exceptions [18], most applications of

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direct SFE mentioned above were based on extraction of a fixed amount of stagnant liquid sample in a high-pressure vessel with supercritical CO_2. In our laboratory, an automated apparatus was constructed for dynamic extraction of a flowing liquid with supercritical CO_2 in an extraction column. The apparatus can be employed for determination of fluid–liquid partition coefficients of analytes at high pressures [19] as well as for analytical extraction, and the latter application has been the subject of this paper. With respect to the analytical relevance of the results, the direct dynamic SFE of a flowing liquid offers some particular benefits. First, after the extraction run has reached a steady state, the composition of the compressed extract becomes constant (i.e. invariant with time). This provides an important advantage over dynamic SFE of a stagnant liquid where the composition of the compressed extract varies with time. The prolonged times needed to extract heavy (low-solubility) analytes from a stagnant liquid sample may lead to stripping of light (high-solubility) analytes from the trapping solvent by the stream of expanding CO_2. Then, the relationship between the sample composition and the composition of the solution of analytes in the trapping solvent becomes uncertain and poorly defined. Second, as the dynamic SFE of a flowing liquid sample can easily be made a continuous process, there is essentially no limit of the sample volume that may be extracted. Consequently, after expansion of the compressed extract, we obtain a relatively concentrated solution of the extracted analytes in the trapping solvent. This feature is highly beneficial for the subsequent chromatographic quantitation.

The primary purpose of the present study was to compare two methods of SFE of aqueous samples, namely, a direct SFE of the sample using the apparatus mentioned, and an indirect procedure involving solid-phase extraction of the analytes from the sample with a subsequent SFE of the solid sorbent (SPE–SFE). To this end, we used wine as a typical example of a very complex liquid mixture. We applied both methods of SFE to an extensive set of wine samples, and quantified the extracted analytes using off-line GC. In order to gain more insight into the relative performance of both methods of SFE and to assess their respective potentials for wine variety identification, the quantitative data from both methods were processed by multivariate statistical techniques. To date, the statistical techniques have been applied to a diverse selection of instrumental methods of wine analysis, including liquid–liquid extraction [20,21], solid-phase microextraction (SPME)–GC [22–25], and GC–MS [26,27]. To our knowledge, however, there has been no previous attempt to combine multivariate statistics with the results of SFE–GC of wines.

2. Experimental

2.1. Wine sample coverage and preparation

A total of 121 samples of white and red wines produced from 21 varieties of *Vitis vinifera* L. grapes were subject to analysis by off-line SFE–GC. The varieties are listed in Table 1, with the last column indicating whether the particular variety was included in the multivariate statistical processing of the results or not. The sample set included 101 samples from 1999 vintage, 11 samples from 1998, six samples from 1997, and three samples from 1996. The wines were collected from local producers in eight vine-growing districts of southern Moravia (south-eastern part of the Czech Republic), namely, Bzenec, Mikulov, Mutěnice, Podluží, Strážnice, Uherské Hradiště, Velké Pavlovice, and Znojmo. The producers guaranteed the varietal purity of the samples provided. In order to stabilise all wines prior to either method of extraction, small amounts (100 mg/l) of sodium azide (Sigma–Aldrich, Prague, Czech Republic) were added to the individual samples.

2.2. SPE–SFE of wines

In parallel with the novel method of direct continuous SFE of wines, the wine samples were also analysed by more conventional SPE–SFE method as a reference. In the SPE step, 3.5 g of Amberlite XAD-7 sorbent (20–60 mesh, Aldrich, Milwaukee, WI, USA) were mixed with a 40-g sample of wine. The particular sorbent/wine mixing ratio resulted from preliminary optimisation experiments. The

Table 1
Wine samples

Variety (original designation)	Variety (international designation)	Number of samples	Multivariate statistics
Aurelius	Aurelius	2	No
Cabernet Sauvignon	Cabernet Sauvignon	1	No
Frankovka	Frankovka	8	Yes
Chardonnay	Chardonnay	9	Yes
Modrý Portugal	Blue Portugal	1	No
Müller-Thurgau	Müller-Thurgau	8	Yes
Muškát moravský	Moravian Muscat	6	Yes
Muškát Ottonel	Muscat Ottonel	1	No
Neuburské	Neuburg	4	No
Pálava	Pálava	3	No
Rulandské bílé	Pinot Blanc	4	Yes
Rulandské modré	Pinot Noir	2	No
Rulandské šedé	Pinot Gris	2	No
Ryzlink rýnský	Rhine Riesling	11	Yes
Ryzlink vlašský	Italian Riesling	9	Yes
Sauvignon	Sauvignon Blanc	11	Yes
Svatovavřinecké	Saint Laurent	5	Yes
Tramín červený	Red Tramin	9	Yes
Veltlínské červené rané	Red Early Veltlin	1	No
Veltlínské zelené	Green Veltlin	17	Yes
Zweigeltrebe	Zweigeltrebe	7	Yes

mixture was stirred with a magnetic stirrer at 70 rpm for 2 h, and then filtered through common filter paper. The sorbent on the filter paper was allowed to pre-dry for 2 h at 30 °C in order to remove most of the residual wine. The moist Amberlite sorbent with sorbed analytes was then loaded into the extraction cell of a supercritical fluid extractor (model Lizard 2000, SEKO-K, Brno, Czech Republic), and extracted dynamically for 45 min at 50 °C and 20 MPa. In order to prevent clogging of the outlet fused-silica restrictor with frozen water entrained from the sorbent, the part of restrictor adjacent to the extraction cell was heated to 150 °C. The released analytes were trapped by bubbling the effluent into ethanol (UV-grade, Merck, Říčany, Czech Republic) at 5 °C. The volume of ethanol in the trapping vial was 4 ml, and ethanol was replenished to a constant volume during the extraction run. Although a more sophisticated technique of analyte trapping into a solvent had recently been developed in this laboratory [28], the more conventional trapping technique was employed here to retain consistency with the direct continuous SFE of wines.

2.3. Direct continuous SFE of wines

Wine samples (170 ml each) were extracted directly with supercritical CO_2 in a packed column operated in a continuous, single-pass, counter-current mode at 50 °C and 20 MPa. Fig. 1 shows a schematic diagram of the essential parts of the extractor. Design details of the in-house-assembled experimental set-up have been described elsewhere [19]. In the present work, the dimensions of fused-silica restrictors were 25 cm×50 μm I.D. in the aqueous phase restrictor and 80 cm×75 μm I.D. in the CO_2-rich effluent restrictor. In counter-current SFE of aqueous media, it is important to maintain a sufficient density differential between the aqueous and the CO_2-rich phases in order to balance the buoyancy and the drag forces acting on the flowing fluids, and to avoid overloading of the extraction column. Under the operating temperature and pressure mentioned above, the density of pure CO_2 calculated from an equation of state [29] was 0.784 g/ml, i.e. it was sufficiently lower than the density of wine to secure a smooth operation of the extraction column in counter-current

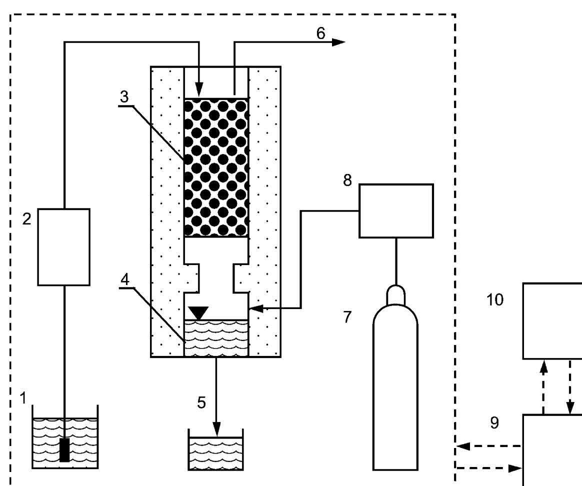

Fig. 1. Schematic diagram of the apparatus for direct continuous extraction of wine samples. 1=wine sample reservoir; 2=wine sample pump; 3=extraction column; 4=phase separator; 5=aqueous phase restrictor; 6=CO_2-rich effluent restrictor; 7=CO_2 cylinder; 8=CO_2 pump; 9=control unit; 10=personal computer for input and display of operating parameters.

mode. The flow-rate of the wine sample through the column ranged within 0.5–0.7 ml/min, and the flow-rate of CO_2 at 50 °C and 20 MPa was approximately 3.7 ml/min. The extracted analytes were trapped by bubbling the effluent into ethanol at 5 °C. The final volume of the solution of extracted analytes in ethanol was approximately 20 ml. In all wine varieties, regardless of whether white or red, the extracts were clear and colourless, indicating that anthocyanins or other heavy, coloured components of wines remained unextracted.

2.4. Quantitative analysis of extracts by GC

The ethanolic extracts from either method of SFE were analysed using a SiChromat 2 gas chromatograph (Siemens, Karlsruhe, Germany) equipped with a DB-WAX capillary column (J&W Scientific, Folsom, CA, USA), 30 m×0.25 mm I.D., with a 0.25-μm coating. The mean linear flow velocity of the helium carrier gas (purity 4.6, oxygen trap, SIAD, Braňany u Mostu, Czech Republic) at the column temperature of 100 °C was 35 cm/s. Nitrogen (purity 5.0, SIAD) was employed as a make-up gas for the flame ionisation detection (FID) system. The 1-μl samples were injected into the column inlet splitter (1:20). Temperatures of the FID system and the injector were 240 and 210 °C, respectively. In the analyses of Amberlite extracts (SPE–SFE), the following temperature program was used: 5 min at 30 °C, then 5 °C/min to 200 °C, then 20 °C/min to 220 °C, and then 1 min at 220 °C. In the analyses of extracts from direct continuous SFE, somewhat slower temperature program was employed: 5 min at 30 °C, then 1 °C/min to 200 °C, then 20 °C/min to 220 °C, and then 1 min at 220 °C. A suitable data acquisition software (CSW v. 1.7, DataApex, Prague, Czech Republic) was employed to collect the detector signal and to evaluate the peak areas.

2.5. Analyte identification by GC–MS

The identification experiments were performed on a mass spectrometer Trio-1000 coupled directly on-line to a Fisons GC 8060 gas chromatograph (Manchester, UK). The spectrometer was operated in the positive electron impact ionization (EI+) scan mode at an ionisation energy of 70 eV. The gas chromatograph was equipped with the same type of column as that mentioned in Section 2.4. The carrier gas was helium (purity 5.5 ECD, SIAD). Splitless injection of 1-μl samples was employed with a split delay of 50 s. Temperatures of the injector and the detector were 200 and 220 °C, respectively. The temperature program used was as follows: 5 min at 30 °C, then 1 °C/min to 200 °C, then 20 °C/min to 220 °C, and then 1 min at 220 °C. The individual analytes were identified employing the apparatus software libraries of EI mass spectra, with a typical probability of match ranging around 95%. In 26 analytes, it was possible to obtain the respective standard compound, and to confirm the analyte identification by comparing the retention times and the mass spectra. Table 2 lists the 26 analytes identified and confirmed in this way; the vapour pressures at room temperature [30] were included to illustrate the analyte volatilities. The identified components were evenly distributed throughout the chromatograms, suggesting the absence of any bias in the selection of identified components with respect to either volatility or molecular size.

Table 2
Wine components identified by GC–MS and confirmed using the respective standard compound

Component	Molecular formula	Vapour pressure at 25 °C (kPa)[a]	Peak no. in Fig. 2
1-Propanol	C_3H_8O	2.73	3
2-Methyl-1-propanol	$C_4H_{10}O$	1.41	4
1-Butanol	$C_4H_{10}O$	0.834	5
2-Methyl-1-butanol	$C_5H_{12}O$	0.483	9 (merged with the following)
3-Methyl-1-butanol	$C_5H_{12}O$	0.383	9 (merged with the preceding)
3-Hydroxy-2-butanone	$C_4H_8O_2$	1.09	14
Ethyl lactate	$C_5H_{10}O_3$	0.325	10
1-Hexanol	$C_6H_{14}O$	0.111	20
Ethyl-3-hydroxypropylether	$C_5H_{12}O_2$	–	21
Ethyl caprylate	$C_{10}H_{20}O_2$	0.0330	27
Acetic acid	$C_2H_4O_2$	2.08	28
Ethyl 3-hydroxybutyrate	$C_6H_{12}O_3$	–	33
2,3-Butanediol	$C_4H_{10}O_2$	0.0146	34
Isobutyric acid	$C_4H_8O_2$	0.257	37
1,3-Butanediol	$C_4H_{10}O_2$	0.00250	38
1,2-Propanediol	$C_3H_8O_2$	0.0271	40
Butyric acid	$C_4H_8O_2$	0.138	43
3-Methylthio-1-propanol	$C_4H_{10}OS$	–	48
Allylacetate	$C_5H_8O_2$	–	49
Propyl formate	$C_4H_8O_2$	10.8	51
Caproic acid	$C_6H_{12}O_2$	0.00567	53
N-(3-methylbutyl)acetamide	$C_7H_{15}NO$	–	56
Phenethyl alcohol	$C_8H_{10}O$	0.00711	58
Diethyl malate	$C_8H_{14}O_5$	0.00310	64
Caprylic acid	$C_8H_{16}O_2$	0.00372	65
Glycerol	$C_3H_8O_3$	0.0000235	76

[a] Calculated from the Antoine equation constants given in Ref. [30].

2.6. Multivariate statistics

The multivariate statistical techniques [31] included discriminant analysis, canonical correlation analysis, and cluster analysis. These techniques were applied in a parallel, coherent and unbiased way to the results from both SPE–SFE–GC and direct SFE–GC. In either case, the input data consisted of a matrix of the individual GC peak areas in the individual wine samples. All the statistical computations were performed using the proper routines of KyPlot spreadsheet software (version 2.0 beta 15, URL: http://www.qualest.co.jp/Download/KyPlot/kyplot_e.htm). In the following discussion of the statistical techniques, the term ‘‘variable’’ refers to the peak area of a particular component of wine in the GC record. The discriminant analysis served to eliminate redundant (i.e. mutually linearly dependent) variables from the input data, and the canonical correlation analysis served to compute the discriminant functions, i.e. the latent factors differentiating among the wine samples. The discriminant analysis required a constant number of samples per wine variety whereas the number of available samples differed widely from one wine variety to another (see Table 1). Therefore, considering the average number of available samples per wine variety, we decided to employ four samples per variety as the basis for the statistical analysis. Consequently, the wine varieties represented by less than four samples had to be eliminated from the statistical processing. In the varieties represented by more than four samples, we used cluster analysis to select the four samples with the highest degree of mutual similarity for representing the particular variety in the subsequent discriminant analysis.

2.6.1. Cluster analysis

The cluster analysis with Ward's amalgamation rule [31] again used the GC peak areas as the input data, and the significance level was 0.05. Application of this selection procedure trimmed the statistical data base to the 12 varieties marked with ''yes'' in the last column of Table 1. Most of the 12 varieties were white wines, with the only red wines being Frankovka, Saint Laurent, and Zweigeltrebe. Expectedly, the four samples representing a particular wine variety in the processing of direct SFE–GC results differed from the four samples representing the same variety in the processing of SPE–SFE–GC results.

2.6.2. Discriminant analysis and canonical correlation analysis

The input data were 59 (in SPE–SFE) or 87 (in direct SFE) peak areas from GC detector records pertaining to each of the 48 wine samples representing the 12 varieties specified in Table 1. The different numbers of peak areas used in the two methods of SFE reflect the fact that only the peaks exceeding the noise level by a factor of 5 or more were included in the statistical processing. Prior to computing the discriminant functions by canonical correlation analysis, it was necessary to check for redundancy among the input variables. In applications of discriminant analysis to large data sets, such a check has generally been performed to avoid computational problems caused by potential ill-conditioning of the variance/covariance matrix. In the present case, the check was actually needed, as there were strong correlations among the individual variables (=wine components). Elimination of the redundant variables was accomplished using forward stepwise discriminant analysis, a step-by-step build-up of the discrimination model by stepwise inclusion of the individual variables in the order of their decreasing contributions to discrimination among the wine varieties. In this process, the F values governing the inclusion or rejection of a variable ranged within 2–6. Essentially, a value of F measures the ratio of the variance among the individual varieties to the average within-variety variance. Once the forward stepwise discriminant analysis was completed and the discrimination model finalised, the discriminant (canonical) functions were determined by canonical correlation analysis. The results of canonical correlation analysis were then employed for an a priori prediction of wine varieties in ''unknown'' samples of wines, i.e. the samples pertaining to one of the 12 varieties (see Table 1) but not included among the 48 samples used to build up the discrimination model.

3. Results and discussion

3.1. Comparison of GC fingerprints of direct SFE vs. SPE–SFE extracts

Fig. 2 shows a sample chromatogram from direct SFE–GC, whereas a record of the same sample from SPE–SFE–GC is shown in Fig. 3. The numbers mark the peaks included in the statistical processing, and they have been assigned in the sequence of elution order. Therefore, a particular number pertains to different substances in Figs. 2 and 3, and the peak numbers listed in Table 2 refer to Fig. 2. It should also be noted that there are different time scales in both figures because of the different temperature programs used in SPE–SFE–GC and in direct SFE–GC (see Section 2.4). The reason for using the different programs was the need to maintain a sufficient resolution in a reasonable time of the peaks exceeding the noise level by a factor of 5 or more because only those peaks were included in the statistical processing. Furthermore, the chromatograms obtained from the individual samples of a particular wine variety were generally more uniform in direct SFE–GC as compared to SPE–SFE–GC. Although useful for the statistical treatments, the complete sets of quantitative GC data are not reported here because they do not provide a direct information on composition of the respective wine samples. In addition to sample composition, the factors influencing the GC records include the thermodynamic and transport properties that control the distributions of the analytes in the separate, multicomponent, two-phase systems involved in the extraction procedure (i.e. the wine–CO_2 system in the direct SFE, the wine–sorbent and sorbent–CO_2 systems in SPE–SFE, and the CO_2–ethanol system in both extraction methods). Regardless of the meth-

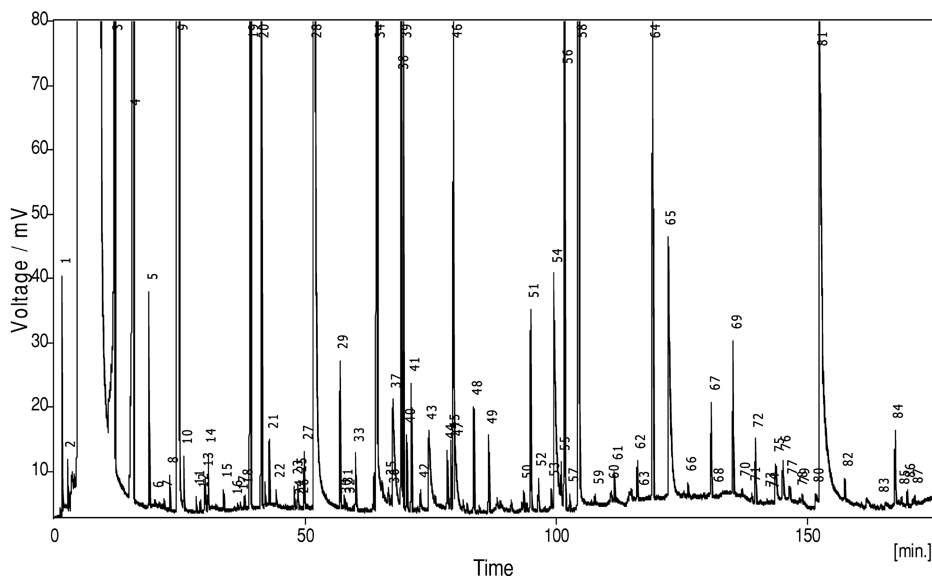

Fig. 2. GC detector record of the direct continuous SFE extract of Rhine Riesling sample (vine-growing region of Velké Pavlovice, 1999 vintage). The components with confirmed identity are listed in Table 2.

od of SFE employed, there were no readily apparent differences in the GC records from white and red wines.

3.2. Statistical analysis of SPE–SFE–GC results

Fig. 4 illustrates the discrimination among the 12

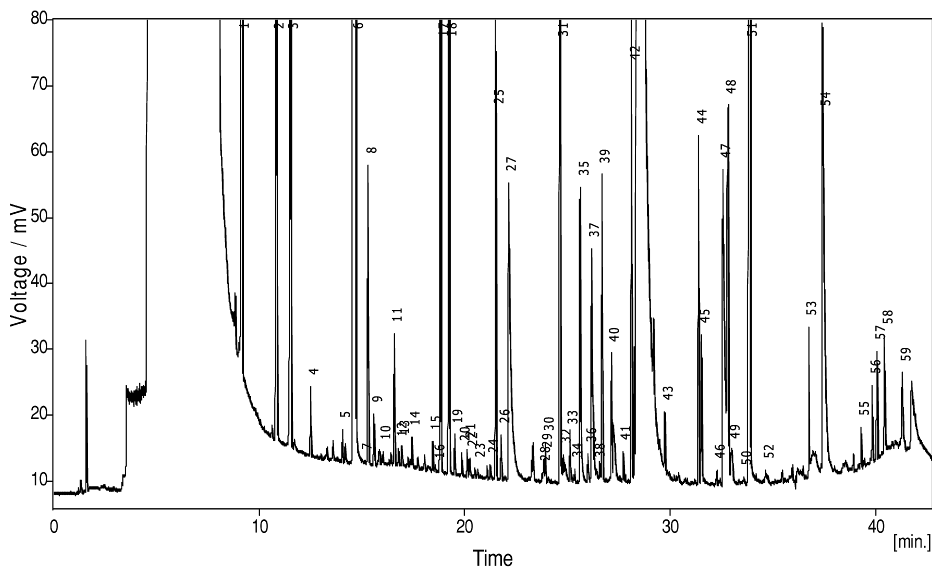

Fig. 3. GC detector record of the SPE–SFE extract of the same wine sample as in Fig. 2.

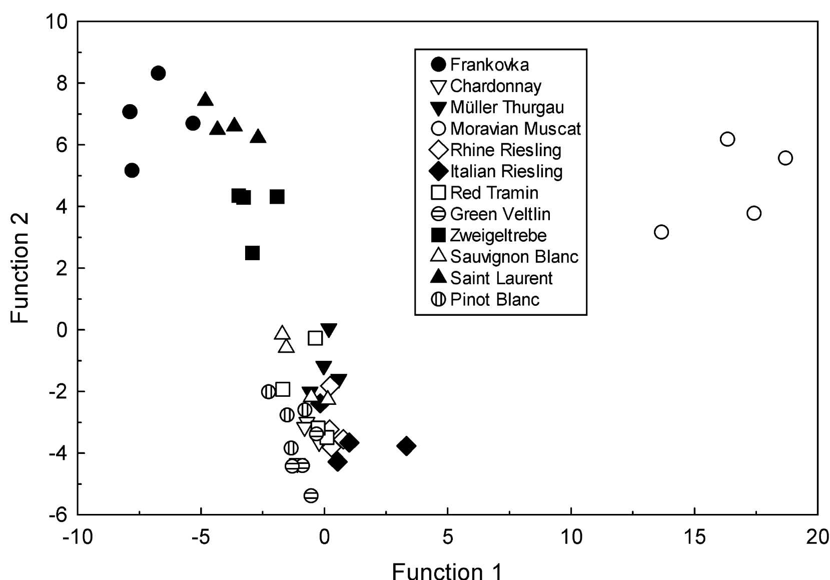

Fig. 4. Discrimination among 12 wine varieties from SPE–SFE–GC. The plot shows the scores of the two most important discriminant functions.

wine varieties (48 samples) from Table 1 as obtained from the two-step process combining solid-phase extraction of wine samples and SFE of Amberlite sorbent. Among the 59 peak areas per sample in the input data, 41 were eliminated by forward stepwise discriminant analysis so that the canonical correlation analysis was based on the remaining 18 variables. The good resolution of the Moravian Muscat samples from the other wines might reflect the strongly aromatic character of this particular variety. Also, the group of three red wine samples (Frankovka, Saint Laurent, and Zweigeltrebe) are well discriminated from the other samples. However, the discrimination among the remaining eight varieties is poor.

3.3. Statistical analysis of direct continuous SFE–GC results

Variety discrimination based on direct SFE–GC is shown in Fig. 5. In this case, the forward stepwise discriminant analysis eliminated 66 of the 87 peak areas per sample in the input data so that the canonical correlation analysis employed the remaining 23 variables. Compared to the results from SPE–SFE, the discrimination among the individual varieties has improved considerably, partly because here there is much less variance within the individual varieties. It should be noted that the improvement could not be an artefact of the statistical processing because there was no systematic difference in the F values used in the forward stepwise discriminant analysis of the direct SFE–GC results and the SPE–SFE–GC results. Again, the red wine varieties are distinctly removed from white wines. Nevertheless, there is still a group of six insufficiently resolved varieties at high scores of discriminant function 1. Therefore, these six varieties were taken out of the data base, and the statistical procedures were repeated with the remaining six varieties only. The results shown in Fig. 6 indicate excellent discrimination with fairly low variances within the individual varieties and a distinct separation between white and red wines. In this case, the forward stepwise discriminant analysis employing an F value of six eliminated 18 of the 24 variables involved, and the subsequent canonical correlation analysis employed the remaining six variables. In order to test the dependence of the discrimination in Fig. 6 on the number of variables involved, the forward stepwise discriminant analysis was repeated using a ‘‘softer’’ F value of 4. This procedure resulted in elimination

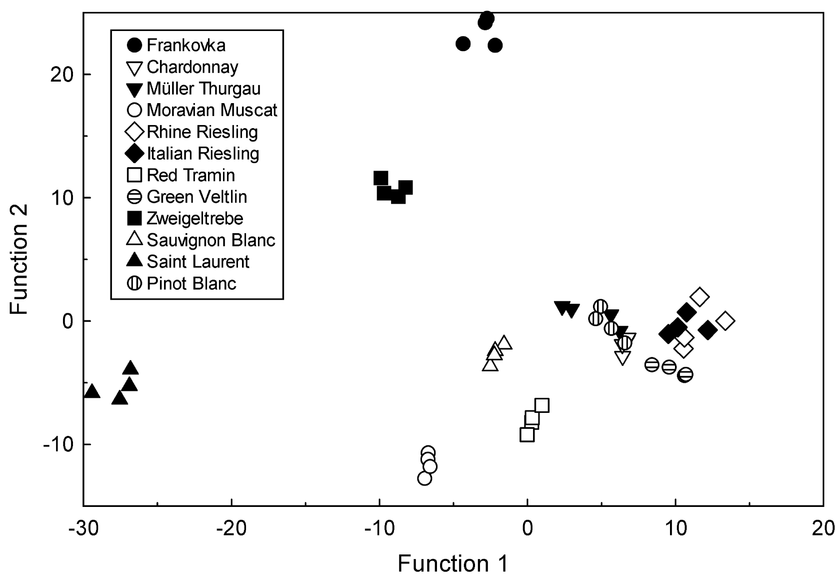

Fig. 5. Discrimination among 12 wine varieties from direct SFE–GC. The plot shows the scores of the two most important discriminant functions.

of 15 of the 24 variables. The subsequent canonical correlation analysis based on the remaining nine variables did not show any apparent improvement in the variety discrimination as compared to Fig. 6. From the perspective of wine variety classification, the results shown in Figs. 5 and 6 present a significant advancement over the application of multivariate statistics to the results of SPME–GC [23–25]

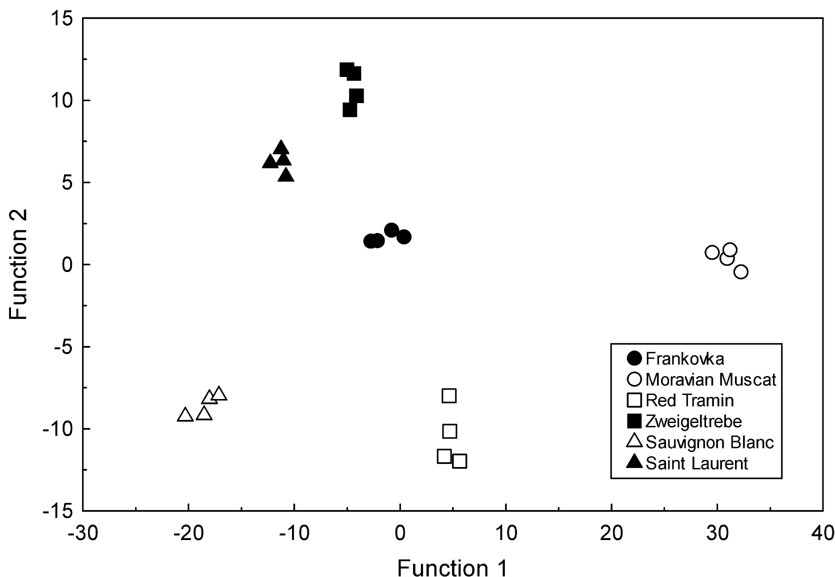

Fig. 6. Discrimination among six wine varieties from direct SFE–GC. The plot shows the scores of the two most important discriminant functions.

in both the quality of discrimination and the number of wine varieties resolved.

3.4. Identification of unknown wine samples

Because of relatively poor overall discrimination among the individual varieties in the discriminant analysis of SPE–SFE–GC results, we did not attempt to use the respective discriminant functions for identification of unknown samples of wine. Instead, employing the direct SFE–GC data, we attempted an identification of 16 unknown samples pertaining to the six wine varieties shown in Fig. 6. Among the 16 samples, there were 10 white wines and six red wines, and the identification resulted in a correct assignment of wine variety in 10 samples (eight white wines and two red wines). Overall, therefore, 63% of identification attempts were successful. It is difficult to state whether this result is satisfactory or not because we are not aware of any previous attempt to identify unknown samples of wines on the basis of extraction/chromatographic data only. In general, however, any identification of wine varieties based exclusively on volatile organic components of wine is certainly less definite than identification based on more complex analytical studies including the inorganic species in wine such as metal ions. The analysis of inorganic species may also be helpful in locating the geographic origin of wine [32].

4. Conclusions

Compared to the two-step process of SPE–SFE, the direct continuous SFE of a wine sample results in a more specific and representative fingerprint of the sample as revealed by the GC analyses of the extracts. Direct SFE is preferable because it is more straightforward than SPE–SFE. The additional analyte–sorbent interactions and sorption/desorption steps involved in SPE–SFE result in unfavourable alteration of the GC fingerprint, reducing both the peak number and the information content of the chromatogram. These undesirable features of the SPE–SFE process are probably caused by diverse degrees of retention (trapping) of the individual analytes in the SPE sorbent.

In addition, this study has shown the feasibility and expediency of direct SFE for the purpose of wine analysis with the goal to classify the samples according to the respective wine varieties. The superior robustness of direct SFE compared to SPE–SFE of wines is apparent from multivariate statistical processing of the analytical results. When processed by the same statistical treatment, the results from direct SFE provide for a strikingly better discrimination among the individual wine varieties than the results from SPE–SFE. This conclusion is independent of the cluster analysis used to pre-select the four samples per wine variety because the pre-selection process was not biased in favour of either mode of SFE. Furthermore, this conclusion is supported by the extensive range of this study (121 wine samples in the original data base).

Unfortunately, the two most important discriminant functions used in Figs. 4–6 cannot be identified with any particular substances present in the wine samples. Instead, the functions are related to the eigenvectors pertaining to the two largest eigenvalues of the covariance matrix built from the peak areas in the GC fingerprints of the individual wine samples. Therefore, as the discriminant functions result from complex algebraic transformation of the original chromatograms, the assignment of a straightforward "chemical" significance to the discriminant functions is prohibitively difficult, if not impossible.

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