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Direct chemical ionization–mass spectrometric profiling of urine in premenstrual syndrome

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Abstract: In search of the pathophysiological background of premenstrual syndrome, direct chemical ionization–mass spectrometric profiling was applied to urine samples from 17 patients and 18 control subjects, collected on days 11 and 25 of the menstrual cycle. Oestrogenic compounds and hippuric acid were found to be involved in differences between these groups. Quotient profiles calculated for each subject from their profiles of days 11 and 25 showed cycle day-dependent group differences. Masses accounting for these differences between patients and control subjects indicate the involvement of unsaturated heterocyclic compounds in premenstrual syndrome.

Keywords: *Premenstrual syndrome; direct chemical ionization–mass spectrometry; profiling; factor-discriminant analysis; quotient profiles; oestrogens; unsaturated heterocyclic compounds.*

Introduction

Premenstrual syndrome (PMS) is characterized by physical, psychological and behavioural symptoms, occurring during the late luteal phase of the menstrual cycle and disappearing at the onset of, or during the first days of menstruation. After its first description [1], considerable effort has been directed towards the clinical characterization and pathophysiological delineation of this syndrome. The available information is mainly of a descriptive nature. Although there is strong evidence that it is related to ovarian function [2, 3], the pathophysiological background of PMS still remains unclear.

Direct chemical ionization–mass spectrometric (DCI–MS) profiling of biological fluids promises to be a powerful tool in the classification of diseases and in the investigation of their pathogenesis. It combines soft ionization with desorption and pyrolysis, and thus enables profiling of matrices containing compounds of different polarities and volatilities over a wide molecular weight range. So far, DCI–MS has successfully been used in the characterization of bacteria [4, 5], algae [6], biopolymers [7], wines and sherries [8], and viral proteins [9]. For evaluation of the complex DCI–MS profiles explorative multivariate data analysis techniques such as factor-discriminant analysis [5, 10] and quotient profile analysis [11] are needed.

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In search of the (bio)chemical basis of PMS, we decided to apply DCI–MS profiling to urine samples of women suffering from PMS.

Experimental

Subjects

Patient selection and the study protocol have been described elsewhere [12–14]. In short, women volunteering in the study were recruited by advertisements and selected on the basis of strictly defined criteria. Classification was based on the increase of complaints in the luteal phase versus those in the follicular phase. Women were considered as being PMS patients if there was a significant difference in symptom scores, using a PMS questionnaire, for at least five out of 10 symptom clusters. The following symptom clusters were regarded as being important for the diagnosis of PMS: crying; depression; headache; irritability; lowered school/work performance; mood swings; painful breasts; feeling swollen or bloated; tension; weight gain. Diagnosis was confirmed by a gynaecologist, who excluded the occurrence of somatic pathology. Controls were matched for age, education or profession, and civil state. In the control group score differences were observed for not more than three of the 10 PMS symptom clusters. Seventeen PMS patients and 18 controls were investigated.

Sample collection

During days 11 and 25 of the menstrual cycle 24-h urine was collected into polyethylene bottles. Throughout the collection period the bottles were kept in a cool and dark place. No preservatives were used. Urine samples were stored at −20°C until analysis.

Sample pretreatment

Urine samples were adjusted to pH 2 with 1 M HCl after centrifugation at 2000*g* for 5 min. A portion of 4–8 ml, depending on the concentration, was applied to C-18 columns (Betron Scientific, No. 607303) which were successively preconditioned twice with 2 ml methanol and twice with 2 ml 10 mM HCl. After absorption of the urine components the column was rinsed twice with 1 ml 10 mM HCl. The components were subsequently eluted with five portions of 0.5 ml methanol. Samples were lyophilized and then dissolved in 200 μl methanol.

Mass spectrometry

Direct chemical ionization–mass spectrometry was performed with a Finnigan MAT 8230 double-focusing mass spectrometer coupled to a Finnigan MAT SS300 data system. Ammonia was used as a reactant gas at an indicated source pressure of 53 Pa; the source temperature was set at 240°C. About 1 μl of the sample solution was applied to the DCI wire. Heating of the DCI wire was performed by linear current programming at a rate of 8 mA s^{-1}. The mass spectrometer operated at a resolution of 1000 and a scan rate of 0.7 s/decade. All samples were measured in duplicate. The spectra obtained in the range of desorption and pyrolysate formation were averaged to one profile; duplicate profiles were subsequently averaged to one individual profile. High-resolution measurements (R = 10 000) were carried out by applying the acceleration voltage scan technique, with tris-(pentafluoroethyl)-s-triazine as a reference compound.

Data analysis

The pattern recognition program ARTHUR [15] extended with routines for principal component-discriminant (PC-D) analysis and graphical rotation (FOM Institute, Amsterdam) [10], was used for data analysis, as well as home-written routines for BIPLOT facilities, for conversion of factor spectra to SS300 mass spectrum formate and for the calculation of quotient spectra [11].

Between-group differences were analysed by averaging individual profiles of days 11 and 25 to one individual profile. Interactions between group and cycle day (cycle day-dependent group differences) were analysed with quotient profiles [11], calculated by dividing the individual profiles of day 11 by those of day 25.

Averaged and quotient profiles were reduced to subsets of 70 variables, the highest Fisher weights being the selection criterion. The resulting patterns were normalized to total ion current for adjustment of differences in sample size, and then transformed by autoscaling (zero mean and unit standard deviation).

Subsequently, PC-D analyses were performed on the transformed data sets. For both data sets the first nine principal components with the highest variance contributions were used as input variables for the discriminant analysis. For display purposes two-dimensional plots were constructed by plotting the discriminant function against an arbitrarily chosen original variable.

Factor spectra obtained after graphical rotation [10], displaying correlations between masses and the discriminant function, were used to investigate the differences in patterns between PMS patients and controls.

The classification performances of the discriminant analyses were evaluated as follows: five subjects (two patients and two controls) were randomly selected from the training set and placed into a test set. Subsequently, feature selection and PC-D analysis were carried out as described above. The classification of the test set subjects was evaluated with a 1-Nearest Neighbour (1NN) routine [16]. These cycles were repeated 30 times, and the mean classification performance was determined.

Results

Group differences

Typical DCI–MS profiles for both categories were obtained by averaging all PMS (Fig. 1A) and control profiles (Fig. 1B). PC-D analysis, performed on the basis of the individual PMS and control patterns, yielded a discriminant function on which both groups showed only minor overlap (Fig. 2). 1NN classification of the test set subjects resulted in a mean correct classification of approximately 63%.

Factor spectra in the 0° (PMS) and 180° (controls) directions revealed some remarkable mass series (Fig. 3). In the 180° spectrum the series *m/z* 288 and 306 may correspond to oestrone ($[M+NH_4]^+$ ion) and estriol ($[M+NH_4]^+$ ion), respectively. The ions at *m/z* 290 may originate from oestradiol ($[M+NH_4]^+$ ion). In addition, the intensity found at *m/z* 304 may point to the corresponding ion of hydroxylated oestrone. These compounds are mainly present in urine as conjugates which decompose during heating of the DCI wire. The masses observed are indicative of relatively high concentrations of oestrogens in urines of the control group.

The intensity at *m/z* 194 in the factor spectrum in the 0° direction can be ascribed to the $[M+H]^+$ of hippuric acid methyl ester, which probably has been formed from the corresponding acid during extraction with and storage in methanol. The intensities at *m/z*

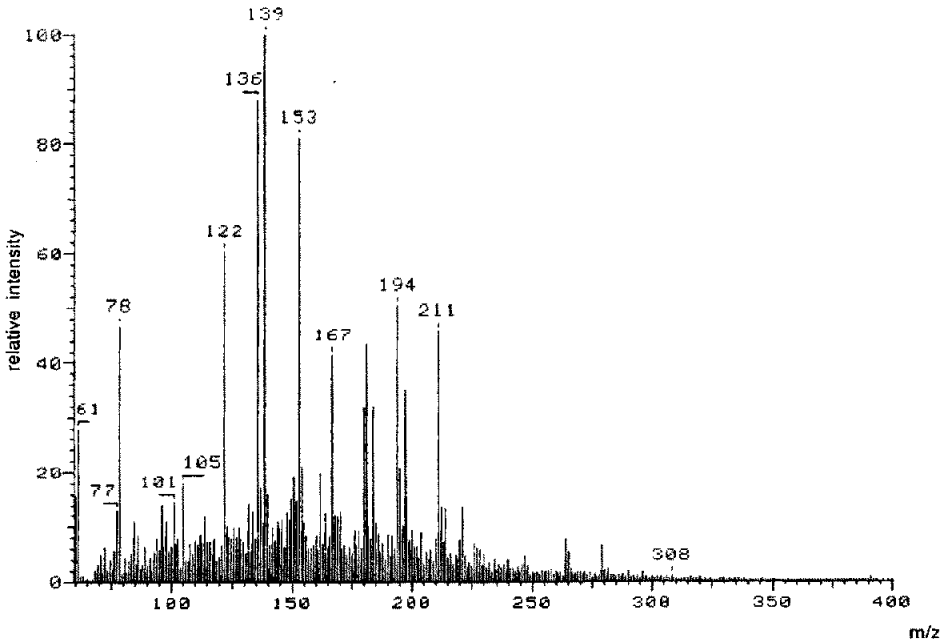

Figure 1A
Averaged DCI–MS urine profile of PMS patients.

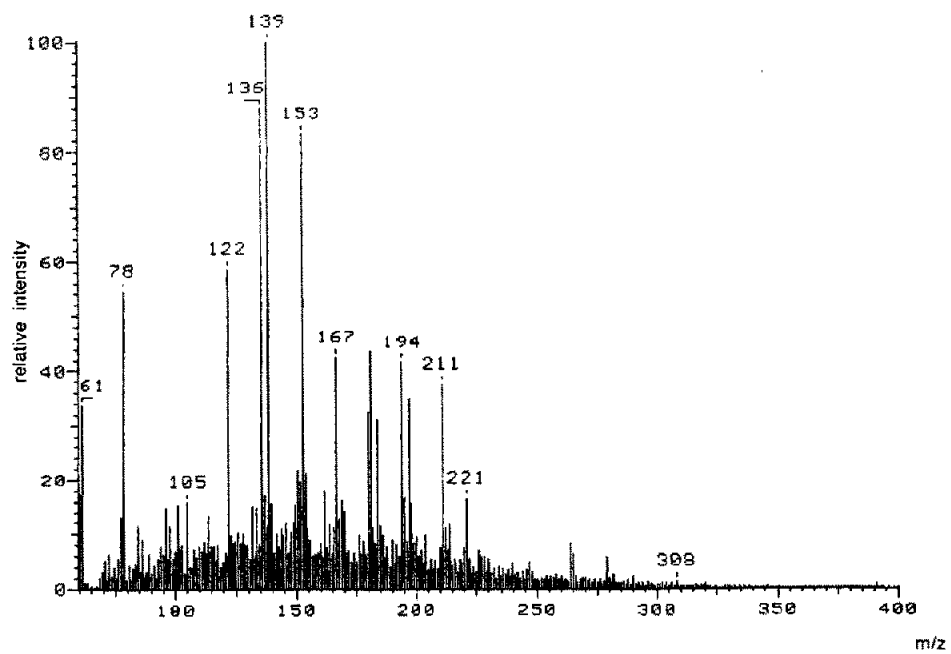

Figure 1B
Averaged DCI–MS urine profile of controls.

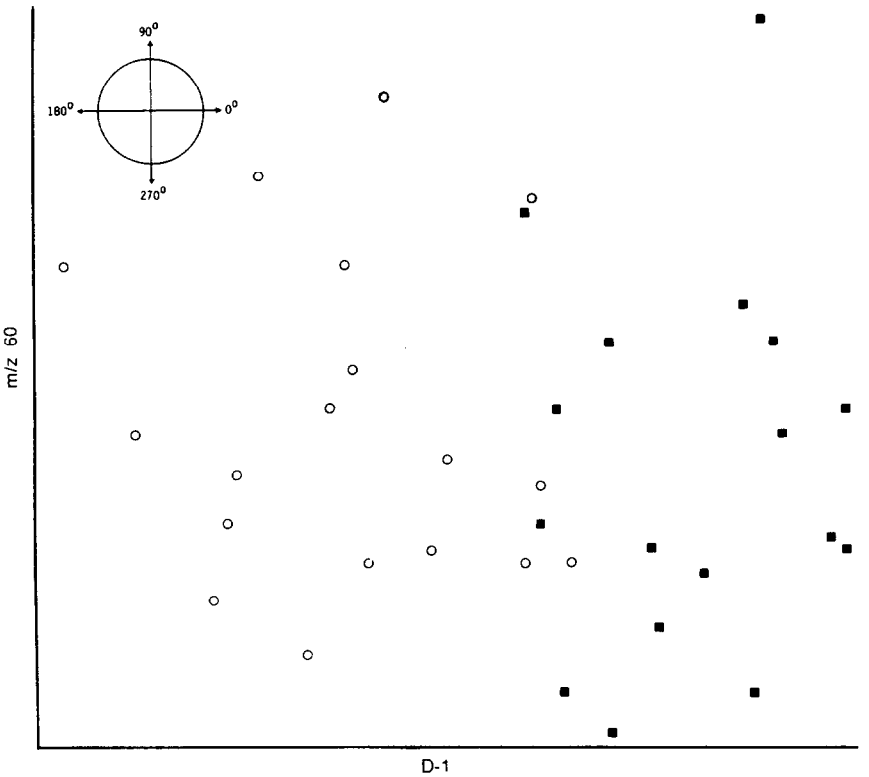

Figure 2
Object plot of a discriminant function versus a random variable introduced for creating a plane. ■, PMS patients; ○, controls.

195 and 196 may be caused by the ^{13}C and ^{18}O isotopic contributions of this compound, represented in the spectrum according to their correlations with the discriminant function. The mass at *m/z* 105 can also be ascribed to the same compound being a benzoyl fragment. This result indicates that this compound was present in relatively high amounts in urine of PMS patients.

A number of masses with comparable correlations with the *D*-axis in the 180° direction have not been identified.

Interaction between group and cycle day (quotient profiles)

To investigate cycle day-dependent differences between PMS patients and controls, the quotient spectrum approach was used [11]. PC-D analysis, applied to the quotient patterns of the control and PMS groups, yielded a discriminant function (Fig. 4), which separated both groups. The 1NN classification performance of the discriminant analysis was approximately 67%.

The factor spectra in the 0° and 180° directions (Fig. 5) revealed a series of masses partly differing from the analysis of the averaged profiles (Fig. 3). A substantial contribution to the spectrum was observed for masses in the higher mass range.

Some of these masses were investigated with high-resolution mass spectrometry providing the following probable elemental compositions: *m/z* 358.140, $C_{18}H_{20}N_3O_5$;

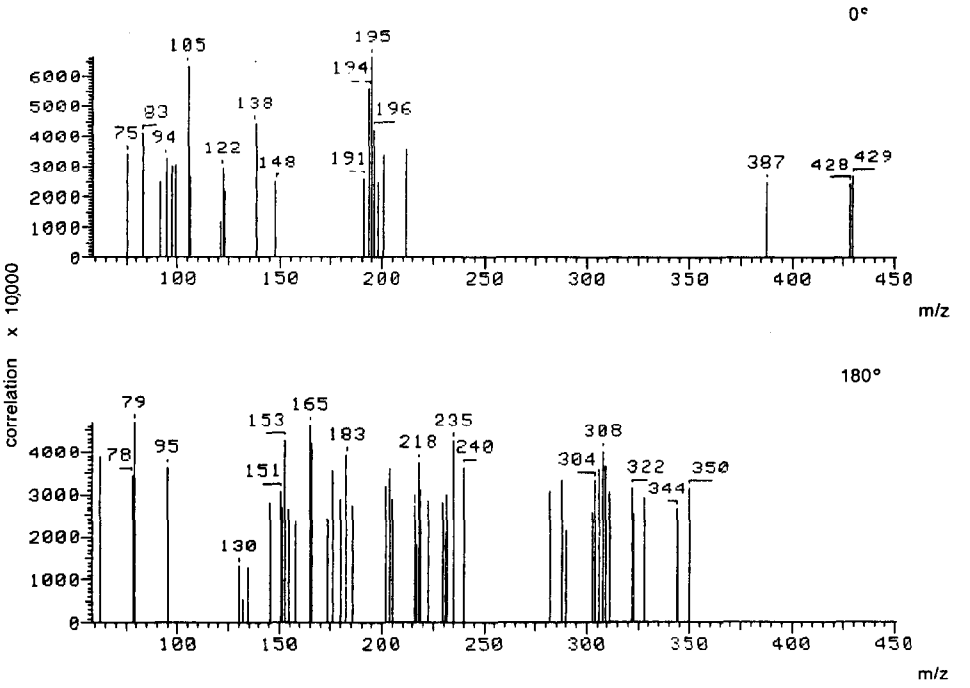

Figure 3
Factor spectra (mass-discriminant function correlations) in the 0° and 180° direction, going together with Fig. 2.

m/z 390.166, $C_{19}H_{24}N_3O_6$; *m/z* 401.173, $C_{18}H_{29}N_2O_6S$; *m/z* 402.167, $C_{20}H_{24}N_3O_6$; *m/z* 415.193, $C_{19}H_{31}N_2O_6S$; *m/z* 420.182, $C_{20}H_{26}N_3O_7$; *m/z* 436.191, $C_{20}H_{28}N_4O_7$.

Because all these compounds contain nitrogen and therefore probably have a higher proton affinity than NH_3, it is likely that preferentially the $[M+H]^+$ ions have been formed. Therefore, the actual elemental compositions contain one hydrogen less. Some chemically relevant differences appeared in the series of elemental compositions: *m/z* 436–420, NH_2; *m/z* 420–402, H_2O; *m/z* 390–358, CH_4O; *m/z* 415–401, CH_2. The elemental compositions point to highly unsaturated, probably aromatic, heterocyclic compounds.

Discussion

In general, the investigation and monitoring of biochemical processes requires a broad analytical approach, because of the complexity and coherence of the phenomena observed. Often a variety of chemical compounds strongly differing in chemical and physical properties is involved in these processes. Most analytical methods are designed for sensitive and specific detection of classes of compounds rather than for providing a general overview of complex processes. For the latter approach the application of profiling techniques can provide new leads to the understanding of pathogenesis. DCI–MS is a profiling technique, that is suitable for the detection of compounds over an extended molecular weight range and with different polarities.

In connection with a non-specific profiling technique, body fluid profiling for the investigation of unknown complex phenomena requires that a sample pretreatment

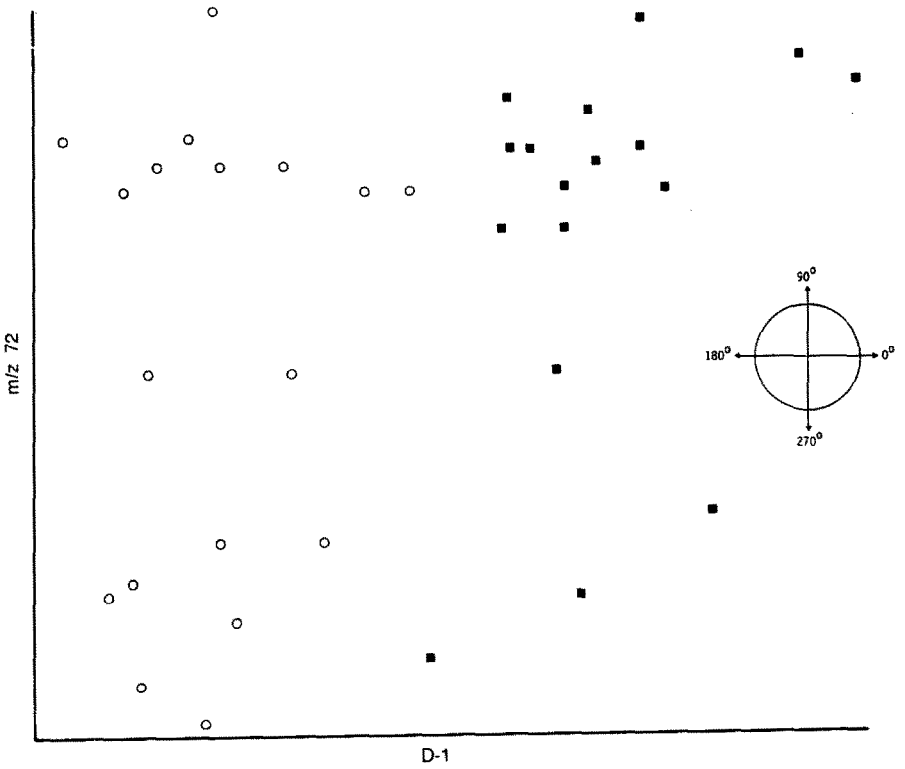

Figure 4
Object plot of a discriminant function, based on quotient profiles, versus a random variable introduced for creating a plane. ■, PMS patients; ○, controls.

method is not very compound-selective. The pretreatment method used was aimed at removing the bulk of salts and urea, while retaining most of the less polar compounds. Therefore it is not unlikely that losses of amphoteric compounds have occurred.

The DCI–MS pattern recognition approach was found to be successful in differentiating between the PMS and control groups, in spite of some overlap due to within-group differences in symptom intensity, symptom heterogeneity and other sources of variation, such as variations in cycle stage and dietary factors.

The main differences observed between PMS and control groups, independent of the cycle day, suggest the involvement of oestrogens and hippuric acid; levels of oestrogens were lower and levels of hippuric acid were higher for the PMS group. The first observation is in accordance with the subnormal peak oestrogen levels that have been reported for PMS patients in the follicular phase [17]. On the other hand, no consistent differences in levels for these compounds were found in the luteal phase [17]. In serum, no significant differences in oestradiol levels were observed between PMS patients and controls [13].

Higher concentrations of hippuric acid in the urine of PMS patients cannot be explained by known metabolic deviations and justify further investigation. The same holds for the coherent series of unsaturated, probably heterocyclic compounds, which were found to be important for differentiation of PMS and control quotient profiles.

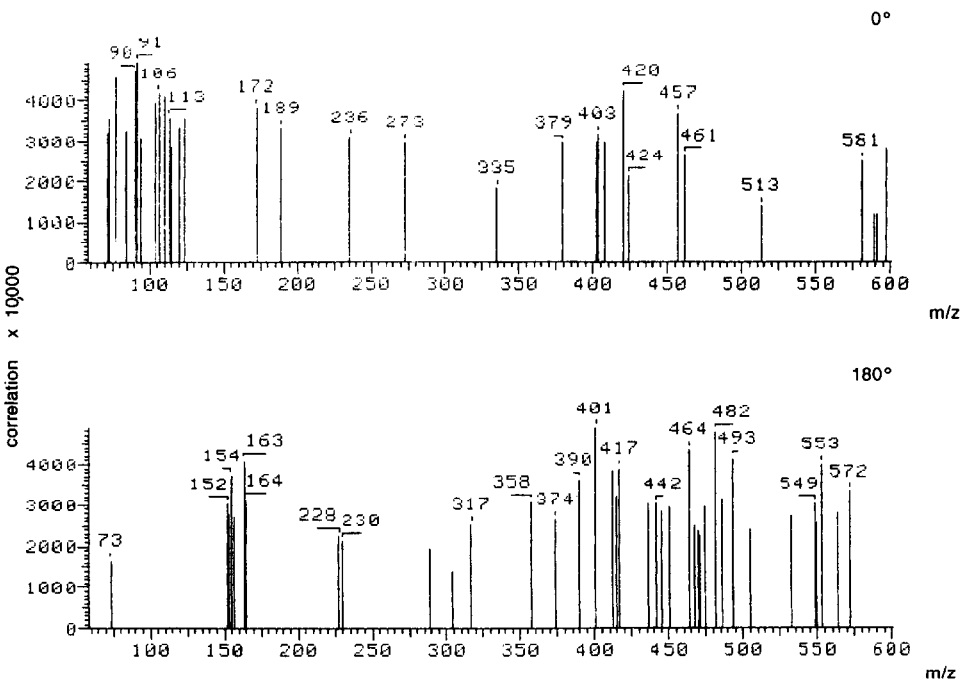

Figure 5
Factor spectra (mass-discriminant function correlations) in the 0° and 180° directions, going together with Fig. 4.

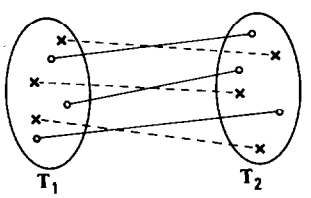

Figure 6
Cluster of objects in a two-dimensional space at two points of time. Although at both events categories are overlapping a clear separation based on trends is possible. The same objects are connected.

Quotient profiles, which can reflect changes in individual profiles with time, are suitable to investigate differences between PMS and controls due to menstrual cycle effects. The principle of this approach is explained in Fig. 6 on the basis of a hypothetical case in which two variables are observed as a function of time for two categories. Apparently, both at time T_1 and time T_2 there is an overlap of both categories, resulting in an incomplete differentiation. However, by focusing on "trend" differences, one can clearly see that all objects of class "X" have decreasing values with time, whereas the class "O" objects have increasing values. In multidimensional data comparable situations exist which can be investigated by this approach.

We conclude that DCI–MS profiling indeed enabled differentiation between women with and without PMS complaints. The profiling technique offers a broad analytic approach which might be useful in the classification of diseases and the investigation of their complex pathogenesis. Additional studies using specific methods focused on the compounds of interest, are required for interpretation of these analytical results.

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