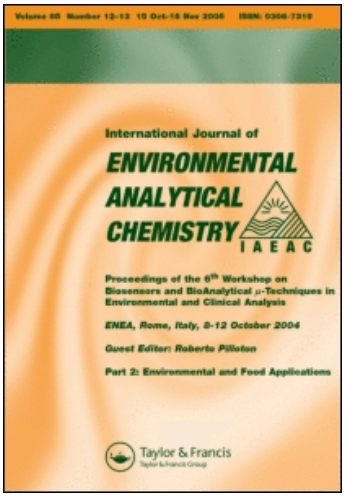

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Statistical Model for Organic Chromatographic Trace Analysis of Complex Samples. A Case Study: Plant Extracts†

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The use of pooled plant extracts is described in the estimation of matrix interference in HPLC (UV and EC) determinations of organic compounds in plant extracts. An extract from freeze dried leaves of 134 different plant species was used for this purpose. It was split in different subgroups with solid extraction clean-up procedures. UV, EC and chromatographic data of the subgroups were used in the calculation of minimum concentrations of organic compounds which are still accurately determinable in plant samples with HPLC methods. The UV and/or EC characteristics of the compound must be known. The contribution of the solid phase extraction procedures and of the analytical system to the selectivity of the method can be estimated. Information is also supplied which allows rapid comparison of the selectivity of the UV and EC (single, or dual parallel) detectors for the determination of a specified compound.

KEY WORDS: Plant extracts, HPLC, matrix interference, UV, EC.

INTRODUCTION

Eighty percent ethanolic extracts from plant material are the starting point for numerous analyses of both endogeneous and exogeneous components. These extracts and subfractions, obtained by using

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clean-up methods, are chemically very complex. As a result, quantitative determinations will be hampered by matrix interference.

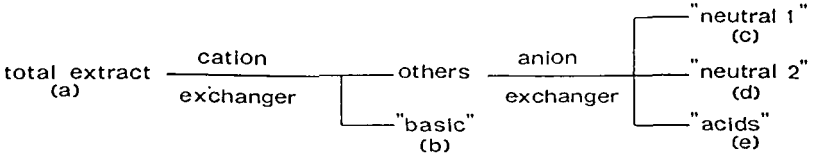
One of the main problems in this area is the lack of quantitative criteria to evaluate matrix interference. Reeve and Crozier[1] suggested a successive approximation method as an evaluation criterion. Purification of the sample to be analysed is continued until an estimate is obtained that does not change on purification. The method is restricted to the evaluation of the determination of a compound in one sample (one plant species) only. Recently, a method was published[2] which predicts matrix interference for the chromatographic trace analysis in extracts from randomly chosen plants. For different clean-up levels, a so called determination limit can be calculated. It is the minimum concentration of a compound (μg per g dry plant material) which can still be accurately determined (relative error due to matrix interferences <0.1) in 9 out of 10 analyses of randomly chosen plants. This limit of determination takes into account matrix interference in a statistical way. The necessary information for its calculation is obtained from physicochemical measurements on a large number of different samples (in our case extracts from different plants). It is the aim of the present study to show how this information can be rapidly obtained using "pooled" extracts.

EXPERIMENTAL

Materials and methods

"Pooled" plant extracts Fresh leaves were collected from trees, shrubs and herbs in the neighbourhood of the Antwerp University. Equal quantities of freeze dried material were taken from each of the 134 collected plant species. These samples were mixed and one gram of the resulting mixture was extracted. It was stirred in 80% ethanol for 24 h, filtered over a Buchner type filter and washed with 20 mL of 80% ethanol. The combined 80% ethanol fractions were evaporated to dryness, the residue dissolved in 10 mL water, and the pH was adjusted to 8.3 with NH_3. After three extractions with diethylether (the ether phase was discarded), the pH was brought to 7 with acetic acid, the volume adjusted to 50 ml, and the sample stored in the deep-freezer.

Subfractions of pooled plant extracts The above pooled total extract (corresponding to 1 g dry plant material) was filtered over a 0.8 μm filter, concentrated, and placed on top of a glass column (1 cm i.d.) filled with a slurry of 10 mL Sephadex SP (H+ form) cation exchanger. Non basic substances are eluted with 80 ml of distilled water. A "basic substances fraction" is eluted with 80 mL 0.2 M NH_3 (see Scheme 1).



SCHEME 1

The non-basic substances fraction is concentrated in vacuo, and applied on top of an anion exchange column (1 cm i.d.) filled with a slurry of 10 mL Sephadex DEAE (formate form). The "neutral 1" fraction (see Scheme 1) was eluted with 80 mL water. The "neutral 2" fraction was eluted with 80 mL 0.01 M ammonium formate. The "acids" fraction was eluted with 80 mL of 4% formic acid. All fractions were concentrated to dryness, dissolved in 5 mL water, the pH adjusted to 3, and stored in the deep-freezer.

HPLC analyses Pooled plant extracts were chromatographed on a reversed phase C8 column of 25 cm length and internal diameter of 4.6 mm, filled with Lichrosorp RP-8 (E. Merck, Darmstadt, F.R.G.). It was eluted with a linear solvent gradient from 5% methanol in 10 mM H_3PO_4 to 80% methanol in 10 mM H_3PO_4, in 40 min (at a flow-rate of 4 mL per min).

The apparatus was a Hewlett-Packard 1084B liquid chromatograph equipped with a variable wavelength detector.

Hydrodynamic voltammograms HDVs of compounds and pooled extracts were obtained as described earlier (ref. 7), with a "continuous" method, i.e. the samples are dissolved in a methanol:10 mM H_3PO_4 eluent (20:80, v:v). This solution is pumped with a Waters type HPLC pump through a Bruker flow-through electrochemical detector at a flow-rate of 2 mL per min. The detector's work

electrode (glassy carbon) diameter is 6 mm. Voltammograms are recorded using a Princeton Applied Research model 364 polarograph, and the signal is sent to a Hewlett-Packard 1000 minicomputer for background subtraction and further processing. A scanning rate of 5 mV per second was used. The response provoked by the samples is expressed as μA divided by the concentration of the pooled extracts (g dry plant material per 1 electrolyte).

RESULTS AND DISCUSSION

Analyses of plant extracts with HPLC gradient methods (as described in the experimental section), result in complex chromatograms. The chromatographic peak patterns are different for each plant. Such extracts are called "open random samples".[2] Their statistical characteristics were described.[3] For the HPLC determination of compounds in such samples determination limits (as defined in the introduction) can be calculated from a simple equation:

$$\text{determination limit} = \bar{a}_{i,s}{:}a_{j,s} \times \text{pd} \times 10^6 \times 20.7\ \mu\text{g per g dry plant material.} \quad (1)$$

This equation was fully explained in reference 4. It is valid for the chromatographic determination of compounds present in plant extracts, using a linear physico-chemical detector. The present study describes practical methods to obtain the different terms of the equation, via "pooled" extracts.

The $\bar{a}_{i,s}{:}a_{j,s}$ term deals with the specificity of the detector towards the compound of interest. It describes the ratio of the mean specific response of the plant extracts, to the specific response of the compound which is to be determined (see Glossary). $\bar{a}_{i,s}$ is dependent on the stage of purification. It is a measure for the amount of interferences which are to be expected in the set of extracts. $\bar{a}_{i,s}$ and $a_{j,s}$ have to be expressed in the same units. For a UV detector, this could be absorptivity, in $1 \times g^{-1} \times cm^{-1}$ (absorbance divided by concentration in g per l, and by light pathway in the cell given in cm). The concentration of the plant extracts is expressed here as g dry plant material extracted, per l. For an EC detector, and for a fluorescence detector, it is difficult to obtain absolute specific responses, as these are instrument dependent. However, Eq. (1) only

requires a ratio of specific responses and this ratio can be measured easily for any experimental set-up. Often, the easiest way to do this is by using the HPLC detector in a flow injection mode and to measure $\bar{a}_{i,s}$:$a_{j,s}$ ratios from peak area:injected quantity measurements. Instead of flow injection methods, one could prefer off-line measurements. For a UV, a fluorescence or an electrochemical detector, this means the recording of respectively a UV spectrum, an exitation emission matrix or a voltammogram.

A mean extract is prepared by mixing (pooling) plant extracts on an equal dry weight basis. For this study, we pooled 134 freeze-dried samples (1 g dry plant material each) from different plants. This mixture was extracted and the extract was taken through several clean-up procedures. At each state $\bar{a}_{i,s}$ values were measured. The clean-up procedure which is used in this study is a "solid phase extraction", an approach which is used more and more in complex samples analysis: see Scheme 1. The subfractions can be classified as "basic", "acid" and "neutral". The neutral fraction was split in two parts: one eluting from DEAE sephadex with water (neutral 1) and one eluting with ammonium formiate (neutral 2). These procedures have been described elsewhere.[4-6]

At each stage of purification, $\bar{a}_{i,s}$ values were measured off-line for two detectors, i.e. a UV and an EC detector: see Figures 1 and 2. Figure 1 shows the UV spectra of the mean plant extract (obtained by pooling 134 extracts) at the different clean-up stages. The "total extracts" plot (curve a) has been reduced 10× relative to the "subfractions" plots (curves b, c, d and e for the basic, neutral 1, neutral 2 and acid fractions respectively). This figure gives information about matrix interferences which are to be expected when plant extracts are analyzed with HPLC/UV methods. As can be expected, interferences are higher at smaller wavelengths. There is a fast rise in the curves below 250 nm. Above 400 nm, interferences are negligible. The group fractionation procedure yields subfractions with reduced $\bar{a}_{i,\lambda}$ values as compared to the total extracts. The ratio of $\bar{a}_{i,\lambda}$ (total extracts):$\bar{a}_{i,\lambda}$ (subgroup) can be seen as a "matrix interference reduction factor". For the "basic", "neutral 1", "neutral 2", and "acids" subfractions, these reduction factors are respectively 19, 4.4, 55, and 6.4, at 280 nm. This simply means that at that wavelength, such fractions contain on the average 19, 4.4, 55, and 6.4 times less potentially interfering material, as compared to the total

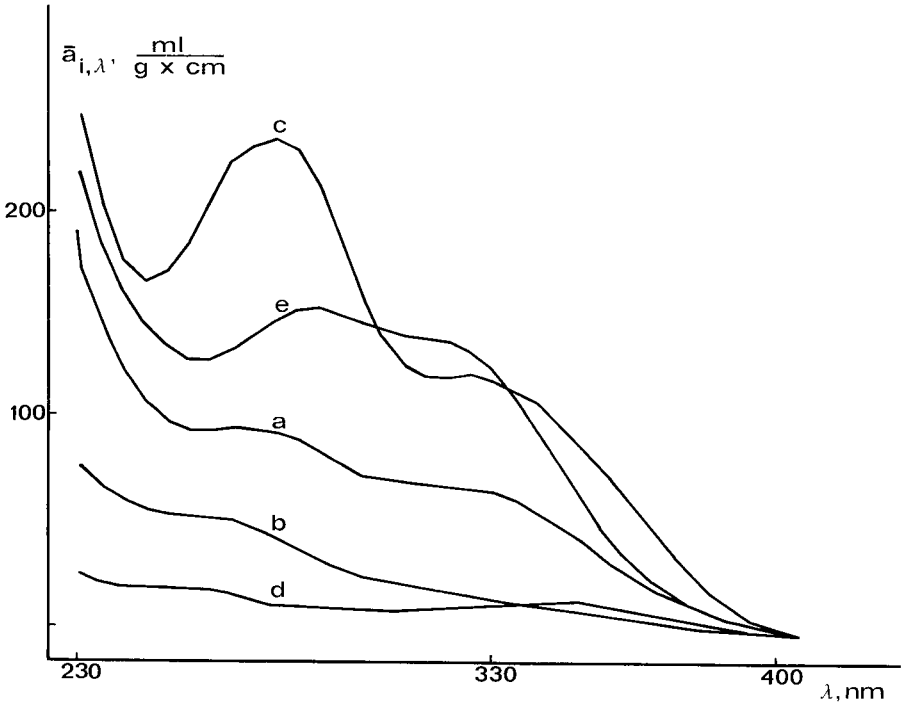


FIGURE 1 UV spectra of the pooled extracts. Curve a represents the total extracts, curves b, c, d and e represent the basic, neutral 1, neutral 2 and acids fractions respectively. Curve a has been reduced 10 times.

extracts. Such reduction factors are useful to express the clean-up efficiency obtained with some newly developed clean-up procedure.

The functions shown in Figure 1 can be used to estimate the determination limit for some compound j, provided its absorptivity is known. This value is substituted in Eq. (1), together with the corresponding $\bar{a}_{i,\lambda}$ value which can be read (with sufficient approximation) from Figure 1. The highest selectivity (smallest $\bar{a}_{i,\lambda}:a_{j,\lambda}$ ratio) is mostly obtained at the wavelength of maximum absorption.[4] If the compound has two λ_{max} values, two determination limits may be calculated. The smaller of the two corresponds to the most selective wavelength. This wavelength should of course be the one used for determination purposes.

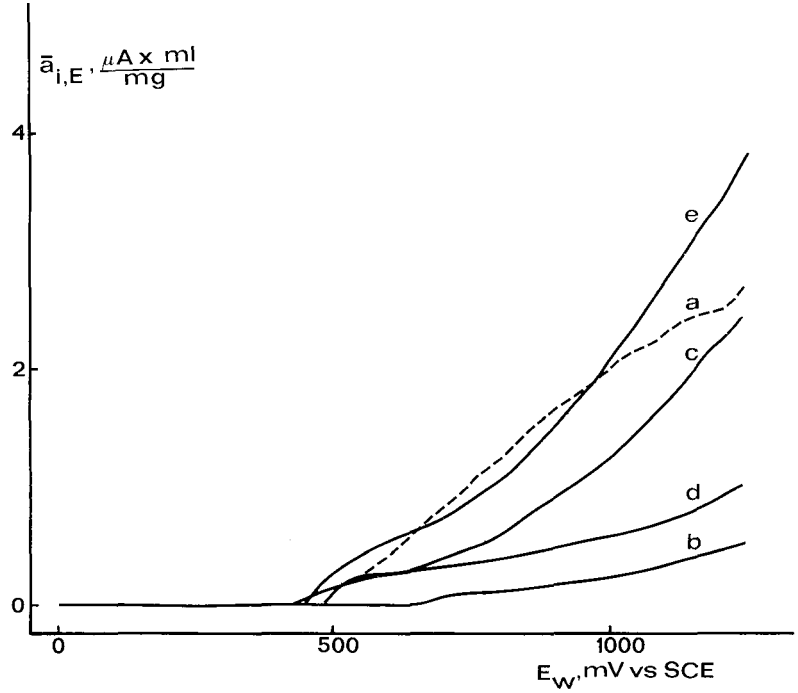


FIGURE 2 Hydrodynamic voltammograms of the pooled extracts. Curves a, b, c, d and e represent the curves for respectively the total extracts, the basic, neutral 1, neutral 2 and acids fractions. Curve a is reduced with a factor of 10.

Specific response curves for the EC detector are shown in Figure 2 for the pooled extracts. These are hydrodynamic voltammograms, obtained from flow injection or continuous measurements (cf. ref. 7 and experimental), with the flow-cell used in chromatographic work. As stated above, absolute specific EC responses are difficult to obtain, as too many factors (cell design, flow rate, eluent composition,...) play a part. This means that the curves of Figure 2 cannot be exactly reproduced on other types of glassy carbon based electrochemical detectors.

The EC detector "sees" practically no components (interferences) under 400 mV (SCE). Above this value, there is a steady increase of matrix interference. Reduction signals were observed at potentials more negative than about −200 mV (SCE) only. We did not observe

negative (reduction) peaks in chromatograms at work electrode potentials above 0 mV (SCE). At potentials above 400 mV, the interferences gradually increase. Typically, at 800 mV (SCE), the group fractionation reduces $\bar{a}_{i,E}$ values with a factor of 105 ("bases" fraction), 19 (neutral 1), 38 (neutral 2) and 12.6 (acids), versus the value of the total extract's response $\bar{a}_{i,E}$. Determination limits for the HPLC determinations of organic compounds in these subgroups will be lowered by this fraction [Eq. (1)] as compared to the determination limits found for the total extracts.

In the experimental conditions used to obtain the HDVs of Figure 2, the plateau limiting current value for catechol (2 electrons transferred) is 550,000 μA:M. The $E_{1/2}$ value for this compound is 730 mV, and the limiting current plateau onset is found at 940 mV. A compound which transfers two electrons to the work electrode will have a specific response that is comparable to this value[7] in our experimental set-up. Compounds transferring 4 electrons will have approximately a doubled plateau current etc.... For other experimental conditions (other flow-cell), one can compare the measured plateau limiting current value for catechol with the above value and deduce a correction factor to calculate $a_{j,E}$ values for electroactive compounds in the own experimental set-up. Using these "normalized" specific responses, and $\bar{a}_{i,E}$ values from Figure 2, one can calculate a determination limit for the compound under study.

Another factor from Eq. (1) which can be obtained from measurements on pooled samples, is the peak density (pd) factor. This factor expresses the separation efficiency of the analytical column for components present in the samples of interest. It is in this respect comparable to optimization functions for multicomponent samples. The peak density pd is the probability that a unit of relative response will be observed in the chromatogram, in a retention time zone from $t_R-2\sigma$ to $t_R+2\sigma$, when a randomly chosen sample is injected.[2] It is obtained from area measurements on chromatograms from extracts pooled on the basis of equal responses (equal absorptivities for a UV detector). This way of pooling extracts is however very unpractical, as it requires separate extraction of each plant, and physico-chemical measurements on each extract. The use of samples pooled on an equal dry weight basis was compared in this respect, as these mixtures are obtained more easily: Equal weights of dry plant material of different plants are mixed and this mixture is extracted.

We experienced no significant difference for peak densities measured with both mixtures. Figure 3a shows the HPLC:UV chromatogram of an injection of the sample obtained by extracting a mixture of dry plant material from 134 different plants (equal weights). Figures 3b, c and d give the same information for the neutral 2, neutral 1 and acids subgroups respectively. From visual inspection of these chromatograms, the spreading of peaks throughout the retention time interval is obtained. The pd values at a retention time t_R are measured from this chromatogram, by dividing the area under the

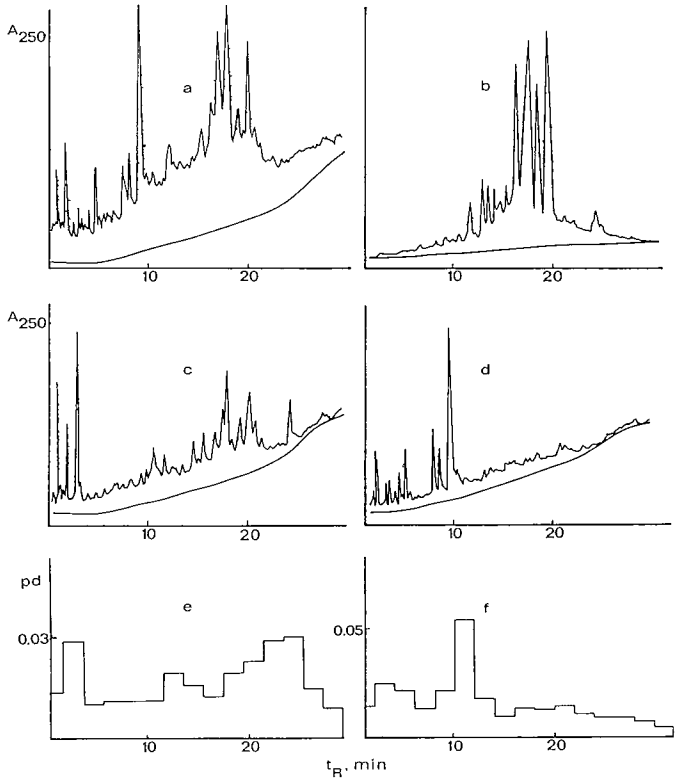


FIGURE 3 HPLC:UV (280 nm) chromatograms on a reversed phase gradient system (see exp.) for pooled total extracts (3a), and respectively the pooled fractions neutral 2 (3b), neutral 1 (3c) and acids (3d). Histograms 3e and 3f are smoothed versions of peak density functions (see text).

graph from $t_R-2\sigma$ to $t_R+2\sigma$ by the total area under the graph. For practical purposes and for calculations [Eq. (1)], it is better to obtain pd values from smoothed graphs: see Figure 3e and f. These show the mean pd values calculated over a larger t_R zone (usually 2.5 to 5σ) for the chromatograms from Figures 3c and 3d respectively.

The spreading of peaks (matrix interferences) over the retention time zone is given in Figure 3a for pooled total extracts. Spreading is quite uniform, indicating good chromatographic selectivity. Locally, i.e. at 9 min retention time, one larger peak appears. This means that at this retention time, the probability of interference is quite high. This peak is due to some very abundant phenolic acids (mainly chlorogenic). The same peak will of course also appear in the chromatogram of the fraction containing the acids (Figure 3d). A bad chromatographic separation efficiency is noted for the "neutral 2" fraction on RP columns, which is badly spread over the retention time interval (Figure 3b). This fraction also contains a few components which seem abundant in most plants, as peak clusters are obtained at retention times of around 18 min. For the neutral 1 and for the acids subfractions (Figures 3c and 3d), there is a reasonably homogeneous spreading of over the entire retention time interval.

Sample pooling is also used in clinical analysis, to obtain an estimate of the interference pattern. In the latter area, there is often a high resemblance of chromatographic peak patterns from sample to sample. As a result, chromatograms from pooled samples will often reflect the single sample's peak patterns. This is not so for the plant samples analyzed in this study. Some non-randomness is noted however for the neutral 2, and for the acids fraction: Chromatograms from pooled acids fractions (Figure 3d) show some sharp peaks in the beginning of gradient conditions. This is an indication that the corresponding compounds may be quite abundant, and likely to interfere in many analyses.

Completely random spreading of peaks over the retention time zone is optimal for good chromatographic analyses. In the latter situation, the peak density will be constant throughout the chromatogram, and equal to the inverse of the peak capacity of the system: $pd = 1:PC$. For good gradient HPLC analyses the peak capacity (for $R_s=1$) is estimated to be 75. The corresponding peak density will then be 0.013. This value can be used as a first estimate in Eq. (1)

when no information is available on the real peak density provided by the analytical system of choice. If, at the retention time of the compound which is to be analyzed, pd would be higher than 0.013, one can eventually decide to develop a more suitable analytical system. The pd factor can be compared to the $\bar{a}_{i,s}$ (total extracts):$\bar{a}_{i,s}$ (subgroup) reduction factors (see above) being also some "interference reduction" factor. It expresses the ratio of the amount of interferences present at the retention time of the compound of interest, divided by the total amount of interferences present in the extracts. In our case, this factor is generally in favour of the analytical system (a median value being 1:75) as compared to the factors noted for the described clean-up methods.

With the data of Figures 1e and 2e, determination limits were calculated for hypothetical compounds, all having a molar absorptivity of 20,000 cm:M, but at different wavelengths, ranging from 250 to 350 nm. The same is done for hypothetical compounds having a specific molar electrochemical response at the limiting current plateau of 470,000 μA:M, also at different E values, ranging from 400 mV to 1,200 mV. The onset of the limiting current plateau is mostly considered as being the optimal potential from the point of view of selectivity.[7] An absorptivity of 20,000 is chosen as this corresponds to a well detectable molecule for the UV detector. A specific electrochemical response of 470,000 μA:M is typical for an organic molecule transferring two electrons to the work electrode[7] (in comparison to UV responses, electrochemical responses are much more quantified and thus more predictable). When molar specific responses $a_{j,s}$ are used in Eq. (1), the determination limit will have units of μmole per gram dry plant material. Figure 4 gives the results of the determination limit calculations for the above described molecules with the UV detector (curve a), for the EC detector with single work electrode (curve b), and for the electrochemical detector with dual electrode parallel (differential) detection (curve c). The three plots are obtained using $\bar{a}_{i,s}$ values (Figures 1e and 2e) for the acids fraction. As can be expected, attainable determination limits depend strongly on the wavelength at which the absorptivity coefficient of 20,000 is observed, and on the potential at which the electrochemical response of 470,000 (onset limiting plateau current) is reached. For most realistic values however, the EC detector will be the more selective (yield the smallest determination limit, see also ref.

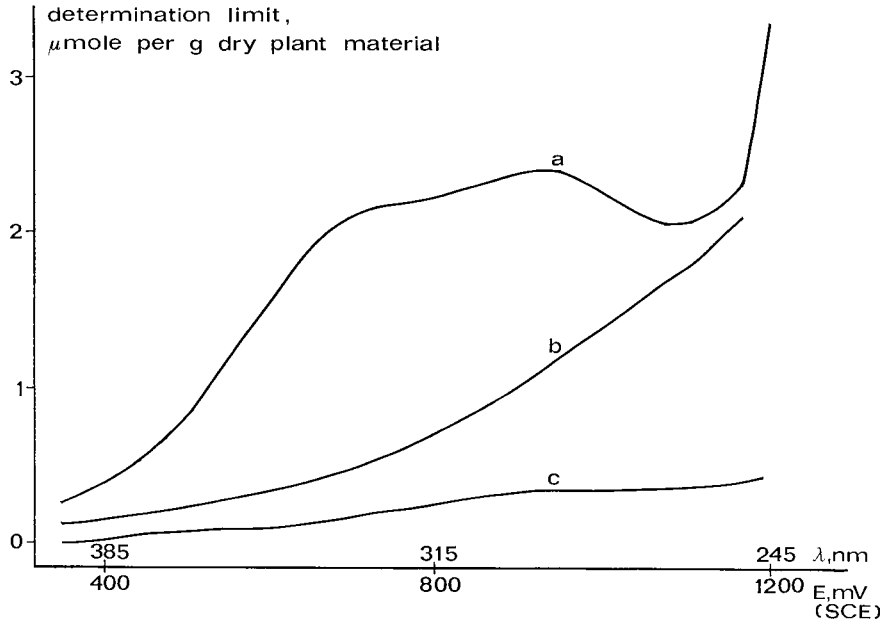


FIGURE 4 Determination limits calculated for hypothetical compounds determined in acids subfractions with HPLC:UV (curve a), HPLC:EC (curve b), and HPLC:EC dual parallel (curve c).

7). Much superior in this respect however, is parallel dual electrode detection, with differential signal ($i1$–$i2$) output. Curve c is calculated for this set-up, with work electrode 1 at the potential of the limiting plateau current onset ($E1$), and work electrode 2 at a potential $E2 = E1 - 90\,\text{mV}$. It was assumed that in these circumstances $E2$ will be located at the start of the voltammographic wave. For these conditions, values of $a_{j,E}$ must be replaced in Eq. (1) by $a_{j,E1} - a_{j,E2}$, and values of $\bar{a}_{i,E}$ by $\bar{a}_{i,E1} - \bar{a}_{i,E2}$. Electrochemical detectors of this type have been described by Kissinger,[8] who also claims them to be less susceptible to noise induced by flow-rate fluctuations as compared to single work electrode detectors. With dual series type electrochemical detectors, such high selectivities will also be obtained. With the latter system, even higher selectivities must be attainable for the determination of compounds showing reversible oxidation reduction behaviour. (The latter detectors have the added

advantage that interfering compounds with irreversible redox characteristics will be "washed out" of the chromatograms).

Knowing the UV and EC characteristics of a compound of interest, one can use Figure 4 to compare the selectivity attainable with both detectors for the HPLC determination of this compound in acids fractions. The results can be extended to other types of extracts which were used in this study, using the information which is given in Figures 1 and 2. For other fractionation schemes, analytical systems, and linear detectors, the preparation of an own pooled sample must be considered. This sample can be taken through the developed clean-up scheme, and data analogous to the data from Figures 1 and 2 can be recorded. Using these data and Eq. (1) a realistic limit of determination can be calculated. This determination limit can be used to evaluate the selectivity of the developed method. As each step in the procedure can also be evaluated, the procedure can be optimized.

CONCLUSIONS

Plant samples pooled on an equal dry weight basis can provide information concerning the quality of HPLC determinations of compounds in plant extracts. Data from UV spectra, HDVs and chromatograms from pooled samples can be used to calculate limits of determination for these analyses. The detector selectivity can be evaluated quantitatively, and the effectiveness of clean-up methods and of the analytical system can be expressed. The method is applicable to HPLC analyses of plant extracts. Extension to other complex samples (biological, environmental etc.) is possible provided the statistical characteristics of the chromatographic patterns of the analyzed samples fulfil certain specifications.

Glossary

PC peak capacity of the chromatographic system. For gradient elution with equal peak width between beginning of gradient ($t=t_b$) and end of gradient ($t=t_e$) conditions, $PC=(t_e-t_b):4\sigma$.

t_R retention time of a compound in the described chromatographic system, in minutes.

$a_{j,s}$ specific response, the response of compound j towards a physico-chemical detector at setting s, divided by its amount or concentration.

$a_{i,s}$ specific response, the response of extract i towards a physico-chemical detector at setting s divided by the concentration of the extract. This concentration is expressed as g dry plant material extracted, per l.

$\bar{a}_{i,s}$ average specific response, the average response provoked by plant extracts i towards a physico-chemical detector at setting s, divided by their concentration (g dry plant material extracted, per l).

pd peak density, the probability that detector responses will be observed in a retention time zone with central value t and width 4σ, when samples are analyzed with the HPLC system.

i current

E potential of an electrode versus a reference. The standard calomel electrode was used in this work.

E_l potential at the onset of the limiting current plateau.

HDV Hydrodynamic voltammogram.

SCE Standard Calomel Electrode.

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