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Fluorometric quantification of alkaloids in the homeopathic mother tinctures of *Vinca minor* L. and *Fumaria officinalis* L.

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To assess the toxic potential of the alkaloids, a quantification method is necessary. An ion pair extraction method was used for quantitative fluorometric determination of vincamine, protopine and all contained alkaloids in the mother tinctures of *Vinca minor* and *Fumaria officinalis*. The non-fluorescent alkaloids were transformed into an ion pair with sodium-9,10-dimethoxy-anthracene-sulfonate and then fluorometrically determined and quantified in this study. The applicable ion pair was extracted in a suitable organic solvent, where dichloromethane has proven to be beneficial. Conditions for the ion pairing and fluorometric quantification are given. The recovery rate was used to investigate the quality of determinability and the influence of the mother tincture matrix. The method was applied to determine the concentration of protopine in the range 0.1 – 15 µg/ml and of vincamine in the range of 0.5 – 20 µg/ml. The limit of detection was < 0.3 µg/ml, and the limit of quantification < 0.9 µg/ml for both alkaloids.

1. Introduction

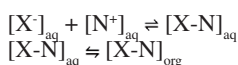
Alkaloids act specifically on different nervous system centers and can lead to varying effects such as stimulation, hallucination, and cytostasis, depending on the dosage. The effect of the alkaloids, which become toxic at too high a dosage, requires a method of quantification for mother tinctures to ensure a pharmaceutical but not toxic effect of preparations. Mother tinctures are plant extracts, which is why the quantity can vary depending on the batch.

Fluorimetry is mainly used for the analysis of physiologically active substances. Due to its high sensitivity, this method can also be used for content determination in low dosages. To make fluorimetry applicable to the mostly non-fluorescent alkaloids, the alkaloids need to be transformed into a fluorescent compound. Fluorescent markers that form an ion pair with the alkaloids are suitable for this transformation (Borg and Westerlund 1970). The method of ion pair extraction of amines in the acidic was studied in more detail by Doyle and Gfeller. Doyle and Levine studied extraction with varying amounts of alkaline nitrogens in the molecule, while Gfeller and Frey tried the method on low dosage pharmaceuticals (Gfeller and Frey 1978; Doyle and Levine 1967). There only have been a few attempts at fluorometric quantification with prior ion pair extraction of amines in pharmaceuticals (e.g. Ahmed and Clark 2007). Fluorimetry combined with ion pair extraction is a promising, rapid, simple, and specific method.

The ion pair extraction is an easy way for the needed transformation of the alkaloids into fluorescent compounds. Since the alkaloids are easily ionized, they build a detectable ion pair using fluorometric counterion sodium-9,10-dimethoxy-anthracene-sulfonate in low pH ranges. Because of the strong fluorescence of the ion pair, the system is highly sensitive, and even low concentrations can be measured.

Ionizable substances are generally applicable to ion pair extraction. To obtain a high degree of selectivity, suitable conditions have to be chosen. The initial solutions must be adjusted to a suitable pH value. With the suitable pH value, the stable ion pair can be extracted with an organic solvent and then measured fluorometrically.

The following equation shows the relation between the concentration of the ion pair in the aqueous phase and the organic phase.



The concentration of the ion pair in the organic phase depends on the concentration of the ion pair in the aqueous phase. The extraction rate depends on the structure of the ion pair. Also, a suitable organic solvent must be used for the extraction (Borg and Westerlund 1970; Gfeller and Frey 1978).

This paper aims to investigate this method to develop an easy but sensitive quantification method for quantifying alkaloids in mother tinctures, specifically in the mother tincture of *Vinca minor* and *Fumaria officinalis*.

2. Investigations, results and discussion

2.1. pH dependence of ion pairing

The equation given in the introduction shows the connection between the aqueous and organic phases. The equilibrium is characterized by the extraction constant. The partition ratio, which describes the interaction between the cation in the aqueous solvent and the ion pair in the organic solvent, can be illustrated with the extraction constant. The shift of the equilibrium can be determined and varied by the concentration of the anion or cation.

To set the equilibrium to the side of the ion pair, the conditions for building the ion pair have to be suitable. Vincamine and protopine can be easily protonated, and sodium-9,10-dimethoxy-anthracene-sulfonate can be deprotonated under acidic pH conditions. To find the suitable pH value for building a stable ion pair, the following measurements were taken:

Solutions of sodium-9,10-dimethoxy-anthracene-sulfonate and vincamine/protopine were prepared at different pH values. After mixing the alkaloid (4 µg/ml), sodium-9,10-dimethoxy-anthracene-sulfonate (200 µg/ml) and dichloromethane in the proportion of 1:2:3, the ion pair was extracted into dichloromethane by vortexing the mixture. The organic phases were fluorometrically measured. The pH values were set with 0.1 M (pH 1), 0.01 M (pH 1.9) sulfuric acid, and different compositions of 0.1 M citric acid monohydrate and 0.1 M trisodium citrate dihydrate (pH 3, 4, and 4.9).

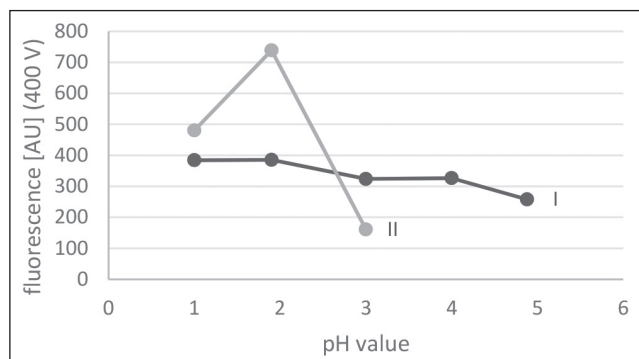


Fig. 1: pH dependence of ion pairing of vincamine [4 µg/ml] (I) and protopine [4 µg/ml] (II) with sodium-9,10-dimethoxy-anthracene-sulfonate [200 µg/ml] – fluorometrically measured in the organic solvent.

As Fig. 1 shows, the ion pair formed from protopine (II) and the counterion has its highest sensitivity and intensity in fluorescence at the pH value of 1.9. Results with the formed ion pair of vincamine (I) and the counterion were not conclusive; the highest fluorescence intensity was shown at pH 1 and pH 1.9.

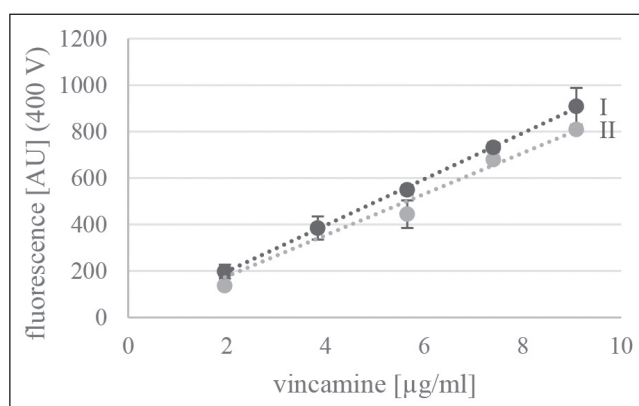


Fig. 2: Fluorescence of dilution series of vincamine in 0.1 M H₂SO₄ (pH 1, I) and 0.01 M H₂SO₄ (pH 1.9, II)

Figure 2 compares the sensitivity of the ion pairing at pH 1 and pH 1.9. Measurements were performed with different vincamine concentrations (2–10 µg/ml) and sodium-9,10-dimethoxy-anthracene-sulfonate in the concentration of 200 µg/ml. The ion pair was extracted into dichloromethane. The calculated linear regression of the measurements made at pH 1 was $f(x)=99.176x$, $R^2 = 0.9999$. In contrast, the function of the measured solutions at pH 1.9 was $f(x)=88.501x$, $R^2 = 0.9952$. As shown in Figs. 1 and 2, the results indicate that the suitable pH value for the ion pairing of sodium-9,10-dimethoxy-anthracene-sulfonate with vincamine is 1 and for ion pairing with protopine is 1.9. Therefore, the solutions were adjusted to pH 1 with 0.1 M or pH 1.9 with 0.01 M sulfuric acid for further measurements.

2.2. Influence of the counterion quantity

To force the partition ratio to rise in a way that the ion pair concentration in the organic solvent increases, the anion concentration must be varied. To ensure that all alkaloid molecules are ion pairing with sodium-9,10-dimethoxy-anthracene-sulfonate, a surplus of the counterion is necessary. The amount of counterion was varied to find the necessary surplus and the smallest possible amount, while still twice as much counterion as alkaloid solution was added.

The results data in Table 1 for comparing the calculated usage of counterion quantity with linear regression clearly pointed to a counterion quantity of 200 µg/ml. Regarding the degree of determination, there is a slight difference between the given counterion concentrations. The sensitivity of fluorescence intensity was highest for both alkaloids at a counterion concentration of 200 µg/ml.

Table 1: Influence of the counterion quantity

	Counterion quantity [µg/ml]	Linear regression	Degree of determination	±SD [%]
Vincamine (2–8 µg/ml)	150	$f(x)=101.94x$	0.9859	0.89 – 10.11
	200	$f(x)=105.48x$	0.9865	1.87 – 8.37
	250	$f(x)=102.03x$	0.9893	1.33 – 7.58
Protopine (1–6 µg/ml)	150	$f(x)=195.58x$	0.9986	1.85 – 8.65
	200	$f(x)=210.85x$	0.9994	2.30 – 5.74
	250	$f(x)=200.43x$	0.9995	2.78 – 7.34

2.3. Influence of the organic solvent

The organic solvent has a strong influence on fluorescence intensity. Measurements with cyclohexane, toluene, ethyl acetate, diethyl ether, methyl tert-butyl ether, chloroform, and dichloromethane as organic solvents were taken.

The fluorescence intensity was around 70 times higher with dichloromethane than with the other organic solvents. Cyclohexane, toluene, ethyl acetate, diethyl ether, and methyl tert-butyl ether are halogen-free organic solvents. Due to the electronegativity of the atoms, they are not polar and do not have a dipole moment. This might be the reason for the low intensity of fluorescence. Because of the component halogen, chloroform and dichloromethane are polar. Chloroform develops a lower dipole moment due to the tetrahedral arrangement of the atoms. The solubility of the ion pair in the organic solvent depends on the polarity of the used organic solvent. On account of the polarity of chloroform and dichloromethane, hydrogen bonding is facilitated. Hydrogen bonds are essential for the solvation of the ion pair. Due to the solvation, the ion pair can be dissolved in the organic phase. Ion pairs usually are defined by their polarity, which leads to a bare dissolvability in non-polar organic solvents. This is why dichloromethane shows the highest fluorescence intensity of all organic solvents tried.

2.4. Optimization of conditions

Due to the high standard deviation within the measurements (Table 1), the conditions needed to be optimized. To optimize the fluorometric quantification method, it was tested whether the standard deviation of the measurement results could be improved by varying the vortex time and phase separation. For this purpose, one part vincamine (6 µg/ml), two parts sodium-9,10-dimethoxy-anthracene-sulfonate (200 µg/ml) and three parts dichloromethane were mixed in a test tube. After the ion pair was extracted into dichloromethane, the test tube was vortexed, and then for the separation of the aqueous from the organic phase, centrifuged at 6,000 rpm for 5 min. There was no difference in the achieved separation efficiency between the centrifuge conditions of 6,000 rpm for 5 min or 3,000 rpm for 10 min. The relative standard deviation was reduced from 3.83 % to 1.98 % for the used alkaloid concentration by increasing the vortex time to 5 min. For phase separation, a relative standard deviation of the measurement results of 3.86 % was obtained by centrifugation and 11.37 % by simply letting the sample stand for 30 min.

2.5. Linearity and sensitivity

Vincamine solutions in 0.1 M sulfuric acid (0.5 – 20 µg/ml) were treated with sodium-9,10-dimethoxy-anthracene-sulfonate (200 µg/ml) and then extracted into dichloromethane (1:2:3 V/V/V). The organic phase was measured fluorometrically. The same procedure was made with protopine solutions in 0.01 M sulfuric acid.

Table 2 shows a good approximation to a linear correlation between the fluorescence intensity and concentration in the examined ranges. Based on the relative standard deviation presented for both alkaloids, the range below 1 µg/ml could be excluded for a reliable measurement. In the range used for determining the calibration curve, the relative standard deviation was between 1 and 7 %.

Table 2: Investigation of the linearity of vincamine and protopine solutions

Considering the concentration [µg/ml] of	Calculated linear regression	Degree of determination	± SD [%]
Vincamine			
0.5 – 1.5	$f(x)=121.35x$	0.9685	9.86 – 29.36
6 – 20	$f(x)=92.56x$	0.9982	3.21 – 4.25
0.5 – 20	$f(x)=92.68x$	0.9975	3.21 – 29.36
After optimizing the method conditions			
1 – 10	$f(x)=131.17x$	0.9956	1.29 – 6.89
Protopine			
0.1 – 1.0	$f(x)=182.66x$	0.9974	4.89 – 25.50
1.0 – 6.0	$f(x)=210.08x$	0.9994	2.30 – 5.74
6.0 – 15.0	$f(x)=193.58x$	0.9977	1.53 – 4.53
0.1 – 15.0	$f(x)=195.50x$	0.9971	1.53 – 25.50

The calculated linear regressions – for protopine ($f(x)=210.08x$, $R^2=0.9994$) and vincamine ($f(x)=131.17x$, $R^2=0.9956$) – were used as calibration curves for calculating the total amount of alkaloids in the mother tincture of *Fumaria officinalis* and *Vinca minor* (2.7.).

2.6. Reproducibility

All measurements were taken multiple times. Each sample was mixed and measured separately. For each concentration, three independent samples were measured. The system's repeatability was determined between 1 and 7 % deviation, depending on the alkaloid concentration, which might be caused by circumstances like room temperature and apparatus deviation. The accuracy of the calibration curve for vincamine was 0.01 – 0.36 µg/ml and for protopine 0.02 – 0.14 µg/ml.

2.7. Fluorometric quantification of alkaloids in homeopathic mother tinctures

The mother tincture of *Vinca minor* was diluted 1:100 with 0.1 M sulfuric acid to set the pH 1. The dilution is necessary to keep the quenching low and take measurements in the linear range of vincamine's calibration curve. The diluted solution was mixed with sodium-9,10-dimethoxy-anthracene-sulfonate (200 µg/ml) and dichloromethane in the proportion of 1:2:3. The formed ion pair of alkaloids with sodium-9,10-dimethoxy-anthracene-sulfonate was extracted into the organic phase while vortexed 5 min and then centrifuged 10 min at 3,000 rpm.

With the final calibration curve with the function $f(x)=131.17x$, the total alkaloids were measured. As a result of the used batch of the mother tincture of *Vinca minor*, the total amount of alkaloids was between 250 and 270 µg/ml.

The necessary degree of dilution of the mother tincture of *Fumaria officinalis* for quantification was determined as 1:200. The mother tincture was diluted 1:200 with 0.01 M sulfuric acid to set the pH to 1.9. For the fluorometric measurement of the alkaloids through the formation of the ion pair, the diluted mother tincture, sodium-9,10-dimethoxy-anthracene-sulfonate and dichloromethane (1:2:3 V/V/V) were vortexed and centrifuged. The organic phase was measured fluorometrically. With the final calibration curve of $f(x)=210.85x$, the total amount of alkaloids was determined between 153 and 163 µg/ml.

2.8. Recovery and matrix effects

The mother tincture of *Bellis perennis* L. (common daisy) represents the matrix of the mother tinctures of *Vinca minor* and *Fumaria officinalis* without alkaloids.

Based on the calculated approximate alkaloid amount in the mother tincture, vincamine was mixed with the mother tincture of *Bellis perennis* with an end concentration of 300 µg/ml of vincamine. The solution was diluted 1:100 with 0.1 M sulfuric acid to set the pH value and keep the quenching low. Separately protopine in the 150 µg/ml concentration was added into the mother tincture of *Bellis perennis* and then diluted 1:200 with 0.01 M sulfuric acid. The alkaloids in the diluted solutions were converted with sodium-9,10-dimethoxy-anthracene-sulfonate (200 µg/ml) into a measurable ion pair for the fluorometric measurement. The ion pair was extracted from the aqueous phase into dichloromethane by vortexing and determined by fluorescence. The extraction and determination were taken on three different days, so the mixture of the mother tincture of *Bellis perennis* and the alkaloid could react, and the matrix effects were recognizable.

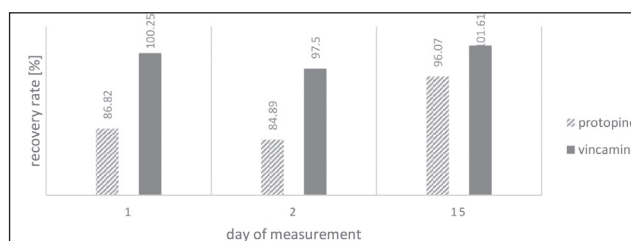


Fig. 3: Recovery rate of vincamine and protopine in the mother tincture of *Bellis perennis* L.

The recovery rates for the different days are shown in Fig. 3. Within those 15 days, the recovery rate of vincamine in *Bellis perennis* could be determined in the deviation of ± 3.2 %. Because of the natural fluctuation and measuring device fluctuation, the measurement led to an almost 100 % recovery rate. The recovery rate of protopine was between 84.89 and 96.07 %. This resulted in an achieved recovery rate of 89 % on average.

2.9. Stability

The stability was tested by analyzing quality control samples at a certain concentration for short-term (50 min) at room temperature and long-term (15 days) at room temperature and 2-8 °C at the refrigerator. The composition of the samples to be measured were as follows: vincamine solution in 0.1 M sulfuric acid (3 µg/ml) or protopine solution in 0.01 M sulfuric acid (4 µg/ml), sodium-9,10-dimethoxy-anthracene-sulfonate (200 µg/ml) and dichloromethane (1:2:3 V/V/V). For short-term stability, the organic phase was measured every 10 min for about an hour. The aqueous and organic phases were not separated; only the required amount of organic solvent was taken for the measurement. For both test series, a total of eight samples were made over three days and measured individually.

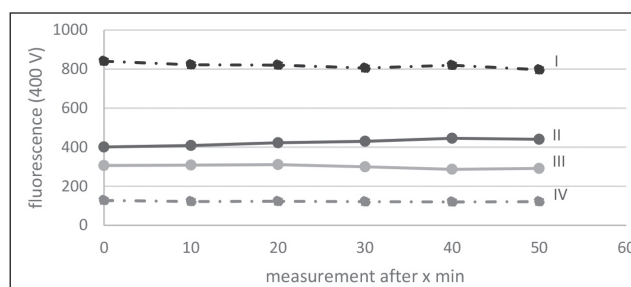


Fig. 4: Intra-day stability of ready-to-measure solutions of protopine [4 µg/ml] (I), vincamine [3 µg/ml] (II), the mother tincture of *Vinca minor* (III) and *Fumaria officinalis* (IV).

Within the first 30 minutes, the samples' fluorescence with vincamine (II) deviated by a maximum of 7.2 % from the initial value (time 0 min). After 50 minutes, the deviation increased up to 11.86 %. While the mother tincture of *Vinca minor* (III) measured

solutions showed only a maximum deviation of 6.83 % within the 50 minutes. The measured baseline fluorescence value deviations were up to a maximum of 6.57 % for the mother tincture of *Fumaria officinalis* (IV) and 5.46 % for protopine (I) within 50 minutes. There was no correlation between an increase in deviation and time of measurement.

The long-term tests showed a significant difference between the storage with and without the aqueous phase and between the storage at room temperature and in the refrigerator. While the relative standard deviation of the stored samples without the aqueous phase led to an increase in fluorescence of more than 370 % within those 15 days, the stored samples with the aqueous phase had a maximum deviation of 77.21 %, which the low boiling point of dichloromethane can easily explain. The samples stored at room temperature with the aqueous phase showed a maximum of 17.58 % fluorescence change within the first three days of measurement. Stored at 2–8 °C, the fluorescence change within the first three measurement days was up to 1.68 % deviation.

3. Experimental

3.1. Materials

Sodium-9,10-dimethoxy-anthracene-sulfonate (Sigma-Aldrich, Steinheim, Germany) was used as the counterion. For the pH adjustment and as an aqueous phase, sulfuric acid (VWR, Darmstadt, Germany) in the concentrations 0.1 and 0.01 M was utilized. The components of pH buffer 3, 4, and 5 were 0.1 M citric acid monohydrate (Merck, Darmstadt, Germany) and 0.1 M trisodium citrate dihydrate (Merck, Darmstadt, Germany). Dichloromethane (VWR, Darmstadt, Germany) was used as the organic solvent.

Vincamine and protopine as a reference substance had a minimum of 98 % purity and was supplied from TCI Chemicals, Tokyo, Japan.

Vinca minor L. and *Fumaria officinalis* L. mother tincture was produced by Deutsche Homöopathie Union, Karlsruhe, Germany, and manufactured after the Regulation 2A of the Homeopathic Pharmacopoeia: Mother tinctures made from fresh plant material.

Only reaction vessels made out of glass were used.

3.2. Instruments and analytical conditions

3.2.1. Apparatus

The fluorometric measurements were performed with a HITACHI Fluorescence Spectrophotometer F-2700 equipped with a 150 W Xenon lamp and a photometric monochromatic light monitoring ration photometry system (Hitachi High-Tech, Tokyo, Japan). The micro-cuvette UBA 400-F10QHM HM-fluorescence cuvette from Uwe Binniger Analytik, Schwäbisch Gmünd, Germany, was used for fluorometric measurements. The extinction slit was adjusted to 2.5 nm and the emission slit to 10 nm. Variations in the instrumental sensitivity were compensated by measuring blank values and by calibration with known solutions.

The suitable wavelength for the counterion sodium-9,10-dimethoxy-anthracene-sulfonate and the ion pair in dichloromethane was in extinction 383 nm and emission 450 nm.

3.2.2. Quality control and statistical analysis

Samples without any alkaloids in them were measured at the same time. Those blank values are necessary to minimize the solvent effect, the impact of ambient humidity and temperature, as well as to calculate the excess of non-ion pairing sodium-9,10-dimethoxy-anthracene-sulfonate in the organic solvent. Data, representative of three independent experiments with similar results, are presented as the average of triplicate observations.

3.2.3. Precision, limit of quantification and limit of detection

Precision was expressed as relative standard deviation (SD %) and did not exceed 7 % for both alkaloids. For measuring blank values, only sodium-9,10-dimethoxy-anthracene-sulfonate, the appropriate aqueous buffer, and dichloromethane were mixed. The organic phase was measured fluorometrically. The limit of quantification and the limit of detection were calculated via the calibration line slope and the standard deviation of the blank values ($LoD_{vincamine} = 0.26 \mu\text{g/ml}$, $LoD_{protopine} = 0.09 \mu\text{g/ml}$; $LoQ_{vincamine} = 0.81 \mu\text{g/ml}$, $LoQ_{protopine} = 0.27 \mu\text{g/ml}$).

3.3. Sample preparation

The samples were prepared as described following: All pH buffers and solvents were previously adjusted to the desired pH value. 0.1 M sulfuric acid was used for pH 1, 0.01 M sulfuric acid for pH 1.9. pH 3–5 were adjusted with different compositions of 0.1 M citric acid monohydrate and 0.1 M trisodium citrate dihydrate. Vincamine and protopine were then separately dissolved in those different pH buffers and solvents. Sodium-9,10-dimethoxy-anthracene-sulfonate was individually dissolved in the same buffer or solvent as the alkaloid. The mother tincture of *Vinca minor* was diluted 1:100 with 0.1 M sulfuric acid, while the mother tincture of *Fumaria officinalis* was diluted 1:200 with 0.01 M sulfuric acid.

In the proportion of 1:2:3 the sample solution of the reference alkaloid or mother tincture, sodium-9,10-dimethoxy-anthracene-sulfonate, and dichloromethane were combined while vortexing 5 min and then centrifuged at 3,000 rpm for 10 min. The organic phase was fluorometrically analyzed.

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Conflict of interests: The authors declare no conflict of interest.

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