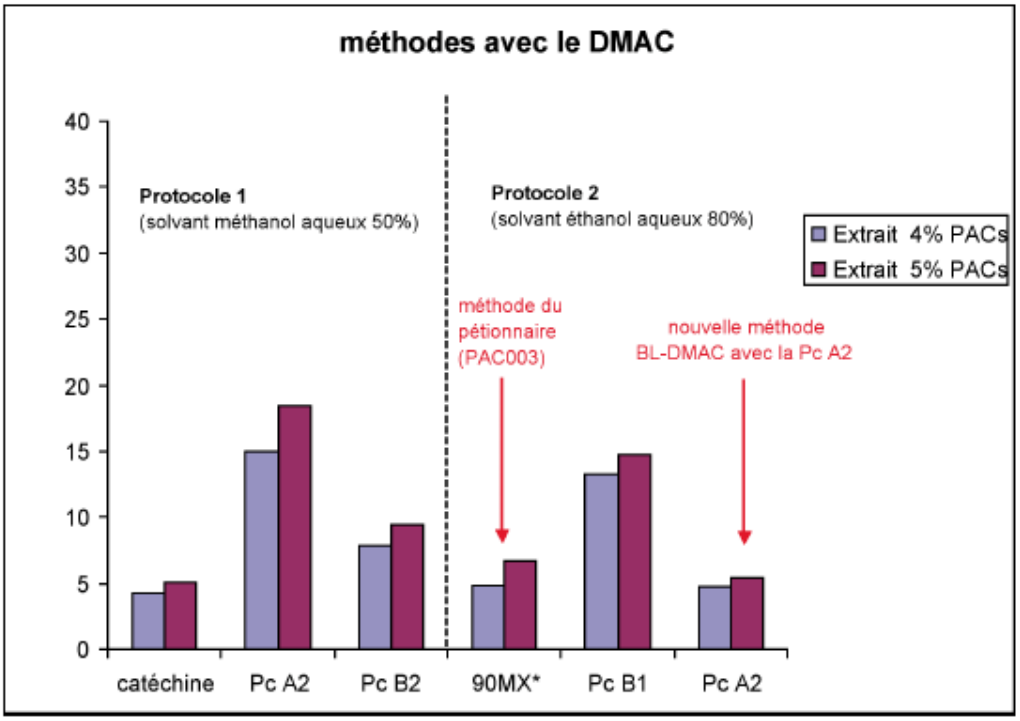
## Cranberries measurements

Proanthocyanidins (PACs), the oligomers and polymers of flavan 3-ols belonging to the flavonoid family, are well known for contributing primary to the astringency flavour and colour of fruit, vegetables and wine[1-3]. But PACs from grape, cocoa, tea, cranberry and many other plants are gaining attention from the medical and pharmaceutical communities for their widely array of potential health benefits[4]. Among these health benefits, PACs found in cranberry (Vaccinium macrocarpon) appear to be of primary importance for prevention of uropathogenic bacterial adhesion[5-9]. In April 2006, AFSSA (French Agency for Food Sanitary Safety) gave a positive opinion on the claim "helps to reduce the fixing of certain E.coli bacteria to the urinary tract walls" and employment of "cranberry" or "Vaccinium macrocarpon" in juice concentrates food supplements and a cocktail/juice nectar. In this claim it is clearly written that the data presented thus suggest that drinking juice of Vaccinium macrocarpon (containing 36 mg of PACs) leads to a decreased incidence of urinary tract infections caused by certain E. coli among adult women[10]. In addition to scientific evidence showing the effectiveness of these ingredients against urinary tract infections, it is necessary to clearly identify and quantify such active compounds (PACs) and link these to the desired health effect. Currently, there is no universally accepted standard method for quantification of PACs in plant or food products. Quantification of PACs is not straightforward and can lead to erroneous, irreproducible results[11, 12]. The complexity of PACs in terms of the large range of molecular weight and linkage types (B-type or A-type) makes it difficult to utilize a single quantification method for all products.



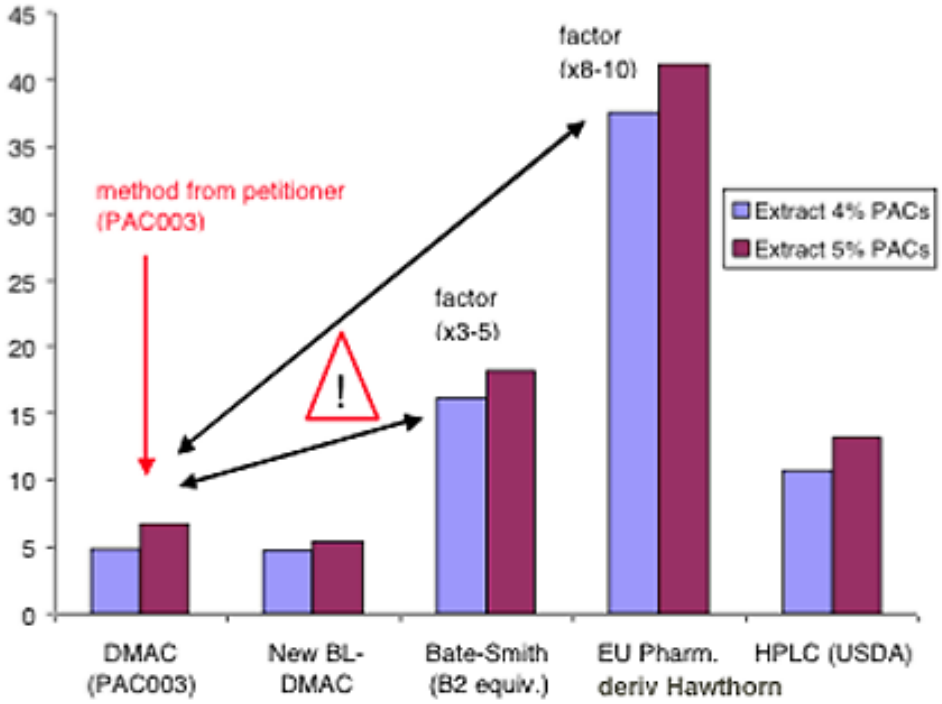

*Dosage des PACs de deux extraits de canneberge utilisant la méthode DMAC mais suivant deux protocoles différents et résultats exprimés selon différents étalons.*
**90MX : poudre de jus de Cranberry contenant exactement 1% de PACs utilisé par le pétitionnaire comme étalon*

Therefore we can classify below analytical methods to measure the concentration in PACs in three groups.

### Methods based on depolymerization of proanthocyanidins

One of the main properties of PACs is to depolymerize under hot and acidic conditions. PACs concentration could be determined by hydrochloric acid-butanol method (Bates-Smith and Porter methods) which oxidatively cleaves inter-flavan bonds to form carbocations from their extension units which are then immediately converted to anthocyanidins[13]. The reaction is greatly influenced by PACs structure, presence of transition metals and complicated by side reactions. The European Pharmacopoeia also uses a method of depolymerization assay to estimate PACs contained in Hawthorn Berries[14]. In both cases, Bate-Smith method or European Pharmacopoeia, the amount of PACs in the sample is expressed as cyanidin chloride. However, we can find derivative methods of Bate-Smith where results are expressed in dimeric or trimeric procyanidin equivalent. In this case, the final concentration of PACs changes again according the standard

used. Moreover, so as to obtain correct result with Bate-Smith or European Pharmacopoeia, it is important to use the proper molar extinction coefficient of cyanidin chloride and carry a white representative sample. Indeed, some plant extracts may already contain anthocyanins, and this is particularly true for the cranberry, which absorb at the same wavelength chosen to measure the colour of cyanidin chloride. In this case, the concentration of PACs may be overestimated. Finally, degrees of polymerization (DPn) and nature of flavan-3-ol units (catechin, epicatechin…) of PACs is achievable by thiolysis or phloroglucinolysis. Thiolysis involves the depolymerization of PACs by breaking the inter-flavan bond in acidic solution. The rupture of this inter-flavan bond causes the release of the terminal unit in its monomeric moiety (catechin or epicatechin) and expansion units as carbocations. The carbocation is very reactive intermediate which can be trapped by a nucleophilic reagent[15]. Thus the thiolysis will identify thanks to the high performance liquid chromatography (HPLC), the qualitative composition of high molecular weight PACs in monomeric units. However, Atype PACs (double linked, see Figure 1) met in cranberry should be resistant




Comparison of analytical methods quantifying PACs on two cranberry extracts standardized at 4% and 5% of PACs using a DMAC method in catechin equivalent

to degradation by thiolysis[16].

## Colorimetric methods

The colorimetric methods use specific reagents reacting with PACs to form a coloured product easily quantified by spectrophotometry UV-VIS. Total phenolic compounds are assayed by the colorimetric methods based on Prussian-blue or using the Folin-Ciocalteu reagent[17, 18]. However, this assay overall phenolic compounds is not specific and therefore not applicable to the determination of PACs. To dose the PACs, more or less specific colorimetric reagents can be used. The older one is the Vanillin method, based on the condensation between the vanillin reagent and the PACs in acidic solutions, leads to a red coloured condensed product. However, with the Vanillin method there maybe other compounds such as flavonols, dihydrochalcones, anthocyanins or ascorbic acid being able to interfere and thus may lead to overestimate the concentration in PACs[19]. So, Vanillin was dropped in favour of DMAC (p-Dimethylaminocinnamaldehyde) yielding more stable and reproducible results. In addition the DMAC reacts specifically with the meta-diphenols to form a green carbonium ion in acidic media and thus does not react with any other flavonoid or with the ascorbic acid [20, 21]. The green adduct formed shows a maximum of absorption at around 640 nm, thus preventing the interference of other compounds that might be present in the same extracts, such as anthocyanins. The DMAC reagent shows a high specifi city for PACs [22].

## Other analytical methods of proanthocyanidins

The method of gravimetry is an ancient method that uses the properties of precipitation of tannins with proteins[23]. So, gravimetric procedures could provide accurate concentrations of total PACs content on larger sample sizes, but, like colorimetric assays, they do not provide qualitative information on molecular weight composition[24]. But the most popular method in phytochemical analysis remains high performance liquid chromatography coupled with detection UV-visible diode array (LC / DAD-UV) or a mass detector (LC/MS) that enable accurate determination of individual molecules and calibration curves can be established when the standards exist. However, in the case of polyphenolic compounds, due to a lack of standards, this technique is rarely used to quantify PACs. Indeed, it is mostly used in qualitative analysis to determine the phenolic composition of a plant extract by identifying different families of phenolic compounds by their UV-VIS spectrum and by their molecular weight when using a mass detector[25]. PACs can be quantified using several assay methods available. But these methods, using different procedures and/or different standards, cannot give the same results for the same product and cannot be correlated between them, at least it is difficult. How are evaluating cranberry extracts dedicated to No. 1924/2006) reminds us to view hundreds of files that are not approved for lack of scientific evidence concerning either the health effects or also the total lack of identification and determination of active molecules related to the health claim. For the cranberry and its health effects on urinary tract infections all does not seem lost, since American scientists will soon published a scientific paper about the analytical method using the DMAC reagent for dosing the PACs. This new DMAC method should correlate the dosage of 36 mg of PACs obtained with the method used by the petitioner by using a modified protocol of the original method (PAC003) and a commercial standard, the procyanidin A2 [26].

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